IFOSFAMIDE AND ASCORBIC ACID MEDIATED ANTICANCER ACTIVITY AND HEMATOLOGICAL TOXICITY IN DALTON'S LYMPHOMA ASCITES BEARING MICE

THESIS

Submitted to the Tamilnadu Dr. M.G.R Medical University,

Chennai

In partial fulfillment of the requirements

For the award of the Degree of

MASTER OF PHARMACY

IN

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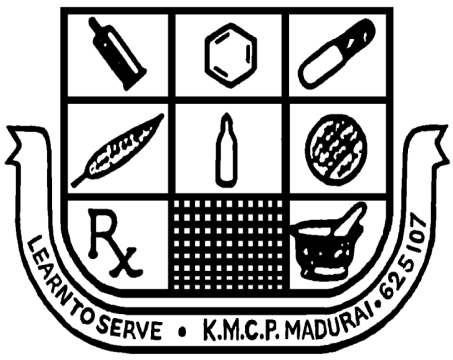

DEPARTMENT OF PHARMACOLOGY

K.M. COLLEGE OF PHARMACY

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APRIL-2015

CERTIFICATE

This is to certify that the dissertation entitled "**IFOSFAMIDE AND ASCORBIC ACID MEDIATED ANTICANCER ACTIVITY AND HEMATOLOGICAL TOXICITY IN DALTON'S LYMPHOMA ASCITES BEARING MICE",** submitted by **Mr. P. MANIKANDAN** in partial fulfillment for the degree of **"Master of Pharmacy in Pharmacology"** under The Tamilnadu Dr. M.G.R Medical University Chennai, at **K.M.College of Pharmacy**, Madurai–107, is a bonafide work carried out by him under my guidance and supervision during the academic year of **2014 – 2015**. This dissertation partially or fully has not been submitted for any other degree or diploma of this university.

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Dedicated to My Beloved Parents, Wife & My Dear Daughter

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CONTENTS

CHAPTER - I

Introduction

CANCER

Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). The branch of medicine concerned with the study, diagnosis, treatment, and prevention of cancer is oncology.

Cancer may affect people at all ages, even fetuses, but the risk for most varieties increases with age.[1] Cancer causes about 13% of all human deaths.[2] Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells.[3] These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited, and thus present in all cells from birth. The heritability of cancers are usually affected by complex interactions between carcinogens and the host's genome. New aspects of the genetics of cancer pathogenesis, such as DNA methylation and microRNAs are increasingly recognized as important.

Genetic abnormalities found in cancer typically affect two general classes of genes. Cancer-promoting oncogenes are typically activated in cancer cells, giving those cells new properties, such as hyperactive growth and division, protection against programmed cell death, loss of respect for normal tissue boundaries, and the ability to become established in diverse tissue environments. Tumor suppressor genes are then inactivated in cancer cells, resulting in the loss of normal functions in those cells, such as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system.

The following closely related terms may be used to designate abnormal growths:

- **Tumor or tumour:** originally, it meant any abnormal swelling, lump or mass
- **Neoplasm:** the scientific term to describe an abnormal proliferation of genetically altered cells. Neoplasms can be benign or malignant:
    - Malignant neoplasm or malignant tumor: synonymous with cancer.

- Benign neoplasm or benign tumor: a tumor (solid neoplasm) that stops growing by itself, does not invade other tissues and does not form metastases.

❖ **Invasive tumor** is another synonym of cancer. The name refers to invasion of surrounding tissues.

❖ **Pre-malignancy, pre-cancer or non-invasive tumor:** A neoplasm that is not invasive but has the potential to progress to cancer (become invasive) if left untreated. These lesions are, in order of increasing potential for cancer, atypia, dysplasia and carcinoma in situ.

CLASSIFICATION:

Carcinoma: Malignant tumors derived from epithelial cells This group represents the most common cancers, including the most common forms of breast, prostate, lung and colon cancer.

Sarcoma : Malignant tumors derived from connective tissue or mesenchymal cells

Lymphoma and leukaemia: Malignancies derived from hematopoietic cells.

Germ cell tumor : Tumors derived from totipotent cells.

Blastic tumor or Blastoma: A tumor (Usually malignant) which resembles an immature or embryonic tissue.

SIGNS AND SYMPTOMS

Symptoms of cancer metastasis depend on the location of the tumor.

Roughly, cancer symptoms can be divided into three groups:

Local symptoms: unusual lumps or swelling (tumor), hemorrhage (bleeding), pain and/or ulceration. Compression of surrounding tissues may cause symptoms such as jaundice (yellowing the eyes and skin).

Symptoms of metastasis (spreading): enlarged lymph nodes, cough and hemoptysis, hepatomegaly (enlarged liver), bone pain, fracture of affected bones and neurological

symptoms. Although advanced cancer may cause pain, it is often not the first symptom.

Systemic symptoms: weight loss, poor appetite, fatigue and cachexia, (wasting), excessive sweating (night sweats), anemia and specific paraneoplastic phenomena, i.e. specific conditions that are due to an active cancer, such as thrombosis or hormonal changes.

PATHOPHYSIOLOGY:

Cancer is a diverse class of diseases which differ widely in their causes and biology. Any organism, even plants, can acquire cancer. Nearly all known cancers arise gradually, as errors build up in the cancer cell and its progeny. Anything which replicates (our cells) will probabilistically suffer from errors (mutations). Cancer is thus a progressive disease, and these progressive errors slowly accumulate until a cell begins to act contrary to its function in the animal.

Mutation:Chemical carcinogens

Cancer pathogenesis is traceable back to DNA mutations that impact cell growth and metastasis. Substances that cause DNA mutations are known as mutagens, and mutagens that cause cancers are known as carcinogens. Particular substances have been linked to specific types of cancer. Tobacco smoking is associated with many forms of cancer,[4] and causes 90% of lung cancer.[5] Prolonged exposure to asbestos fibers is associated with mesothelioma[6].

Mutation: ionizing radiation

Sources of ionizing radiation, such as radon gas, can cause cancer. Prolonged exposure to ultraviolet radiation from the sun can lead to melanoma and other skin malignancies.[7]

Viral or bacterial infection

Some cancers can be caused by infection with pathogens.[8] Many cancers originate from a viral infection; this is especially true in animals such as birds, but

also in humans, as viruses are responsible for 15% of human cancers worldwide. The main viruses associated with human cancers are human papillomavirus, hepatitis B and hepatitis C virus, Epstein-Barr virus, and human T-lymphotropic virus.

Hormonal imbalances

Some hormones can act in a similar manner to non-mutagenic carcinogens in that they may stimulate excessive cell growth. A well-established example is the role of hyperestrogenic states in promoting endometrial cancer.

Immune system dysfunction

HIV is associated with a number of malignancies, including Kaposi's sarcoma, non-Hodgkin's lymphoma, and HPV-associated malignancies such as anal cancer and cervical cancer.

Heredity

Most forms of cancer are sporadic, meaning that there is no inherited cause of the cancer. There are, however, a number of recognised syndromes where there is an inherited predisposition to cancer, often due to a defect in a gene that protects against tumor formation. Famous examples are:

- certain inherited mutations in the genes BRCA1 and BRCA2 are associated with an elevated risk of breast cancer and ovarian cancer
- tumors of various endocrine organs in Multiple Endocrine Neoplasia (MEN types 1, 2a, 2b)
- Li-Fraumeni syndrome (various tumors such as osteosarcoma, breast cancer, soft tissue sarcoma, brain tumors) due to mutations of p53
- Turcot syndrome (brain tumors and colonic polyposis)
- Familial adenomatous polyposis an inherited mutation of the APC gene that leads to early onset of colon carcinoma.
- Hereditary Nonpolyposis Colorectal Cancer (HNPCC, also known as Lynch syndrome) can include familial cases of colon cancer, uterine cancer, gastric cancer, and ovarian cancer, without a preponderance of colon polyps.

- Retinoblastoma, when occurring in young children, is due to a hereditary mutation in the retinoblastoma gene.
- Down syndrome patients, who have an extra chromosome 21, are known to develop malignancies such as leukemia and testicular cancer, though the reasons for this difference are not well understood.

Biological properties of cancer cells

In a 2000 article by Hanahan and Weinberg, the biological properties of malignant tumor cells were summarized as follows:[9]

- Acquisition of self-sufficiency in growth signals, leading to unchecked growth.
- Loss of sensitivity to anti-growth signals, also leading to unchecked growth.
- Loss of capacity for apoptosis, in order to allow growth despite genetic errors and external anti-growth signals.
- Loss of capacity for senescence, leading to limitless replicative potential (immortality)
- Acquisition of sustained angiogenesis, allowing the tumor to grow beyond the limitations of passive nutrient diffusion.
- Acquisition of ability to invade neighbouring tissues, the defining property of invasive carcinoma.

CELL BIOLOGY & CANCER:

Cancer is a group of more than 100 diseases that develop across time and involve the uncontrolled division of the body's cells. Although cancer can develop in virtually any of the body's tissues, and each type of cancer has its unique features, the basic processes that produce cancer are quite similar in all forms of the disease.

All of the cells produced by division of this first, ancestral cell and its progeny also display inappropriate proliferation. A tumor, or mass of cells, formed of these abnormal cells may remain within the tissue in which it originated, or it may begin to invade nearby tissues. An invasive tumor is said to be malignant, and cells

shed into the blood or lymph from a malignant tumor are likely to establish new tumors (metastases) throughout the body.

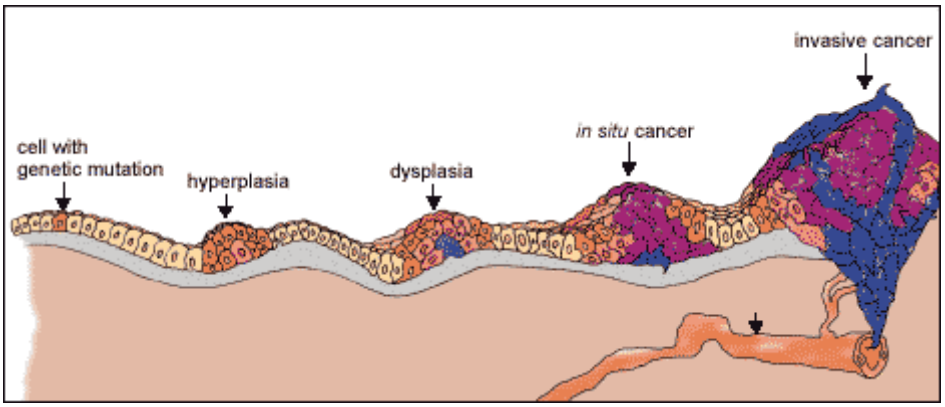
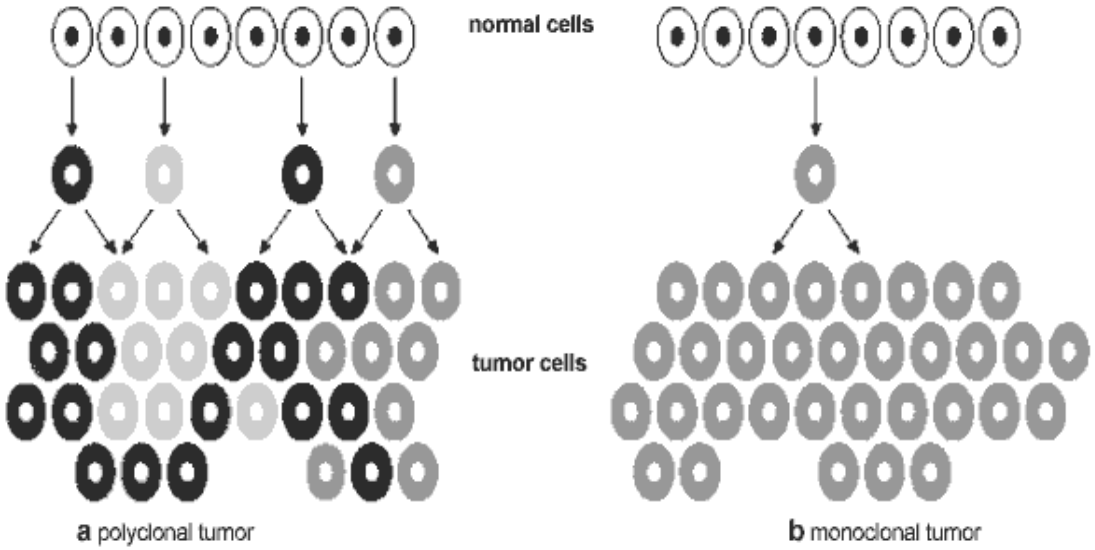


Figure No.1 - The stages of tumor development. A malignant tumor develops across time, as shown in this diagram. This tumor develops as a result of four mutations, but the number of mutations involved in other types of tumors can vary. We do not know the exact number of mutations required for a normal cell to become a fully malignant cell, but the number is probably less than ten. a. The tumor begins to develop when a cell experiences a mutation that makes the cell more likely to divide than it normally would. b. The altered cell and its descendants grow and divide too often, a condition called hyperplasia. At some point, one of these cells experiences another mutation that further increases its tendency to divide. c. This cell's descendants divide excessively and look abnormal, a condition called dysphasia. As time passes, one of the cells experiences yet another mutation. d. This cell and its descendants are very abnormal in both growth and appearance. If the tumor that has formed from these cells is still contained within its tissue of origin, it is called in situ cancer. In situ cancer may remain contained indefinitely. e. If some cells experience additional mutations that allow the tumor to invade neighboring tissues and shed cells into the blood or lymph, the tumor is said to be malignant. The escaped cells may establish new tumors (metastases) at other locations in the body.

Figure No.2 - Two schemes by which tumors can develop. Most—if not all—human cancer appears to be monoclonal



Source: Modified from Varmus, H., & Weinberg, R.A. 1993. *Genes and the biology of cancer.* New York: Scientific American Library.

CANCER: CELL CYCLE

The cell cycle, or cell-division cycle, is the series of events that take place in a cell leading to its division and duplication (replication).

The cell cycle consists of five distinct phases: G_1 phase, S phase (synthesis), G_2 phase (collectively known as interphase) and M phase (mitosis). M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two daughter cells, and cytokinesis, in which the cell's cytoplasm divides forming distinct cells. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G_0 phase.

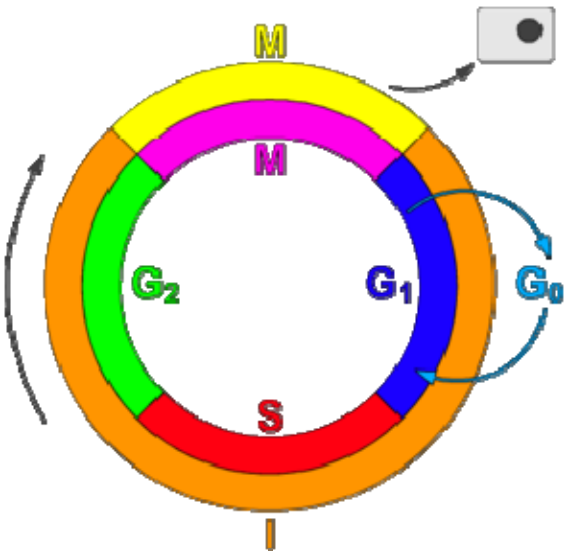


Figure No.3- Schematic of the cell cycle. outer ring: I = Interphase, M = Mitosis; inner ring: M = Mitosis, G_1 = Gap 1, G_2 = Gap 2, S = Synthesis; not in ring: G_0 = Gap 0/Resting. The duration of mitosis in relation to the other phases has been exaggerated in this diagram.

Table No.1

State	Phase	Abbreviation	Description
quiescent/ senescent	Gap 0	**G_0**	A resting phase where the cell has left the cycle and has stopped dividing.
Interphase	Gap 1	**G_1**	Cells increase in size in Gap 1. The G_1 checkpoint control mechanism ensures that everything is ready for DNA synthesis.
	Synthesis	**S**	DNA replication occurs during this phase.
	Gap 2	**G_2**	During the gap between DNA synthesis and mitosis, the cell will continue to grow. The G_2 checkpoint control mechanism ensures that everything is ready to enter the M (mitosis) phase and divide.
Cell division	Mitosis	**M**	Cell growth stops at this stage and cellular energy is focused on the orderly division into two daughter cells. A checkpoint in the middle of mitosis (Metaphase Checkpoint) ensures that the cell is ready to complete cell division.

Resting (G_0 phase)

The term "post-mitotic" is sometimes used to refer to both quiescent and senescent cells. Nonproliferative cells in multicellular eukaryotes generally enter the quiescent G_0 state from G_1 and may remain quiescent for long periods of time, possibly indefinitely (as is often the case for neurons).

G_1 phase

The first phase within interphase, from the end of the previous M phase until the beginning of DNA synthesis is called G_1 (G indicating gap). During this phase the biosynthetic activities of the cell, which had been considerably slowed down during M phase, resume at a high rate. This phase is marked by synthesis of various enzymes that are required in S phase, mainly those needed for DNA replication. Duration of G_1 is highly variable, even among different cells of the same species.

S phase

The ensuing S phase starts when DNA synthesis commences; when it is complete, all of the chromosomes have been replicated, i.e., each chromosome has two (sister) chromatids. Thus, during this phase, the amount of DNA in the cell has effectively doubled, though the ploidy of the cell remains the same. Rates of RNA transcription and protein synthesis are very low during this phase. An exception to this is histone production, most of which occurs during the S phase.[10,11,12]

G_2 phase

The cell then enters the G_2 phase, which lasts until the cell enters mitosis. Again, significant protein synthesis occurs during this phase, mainly involving the production of microtubules, which are required during the process of mitosis. Inhibition of protein synthesis during G_2 phase prevents the cell from undergoing mitosis.

Mitosis (M Phase)

The relatively brief M phase consists of nuclear division (karyokinesis) and cytoplasmic division (cytokinesis). In plants and algae, cytokinesis is accompanied by the formation of a new cell wall. The M phase has been broken down into several distinct phases, sequentially known as prophase, Prometaphase, metaphase, anaphase and telophase leading to cytokinesis.

Regulation of eukaryotic cell cycle

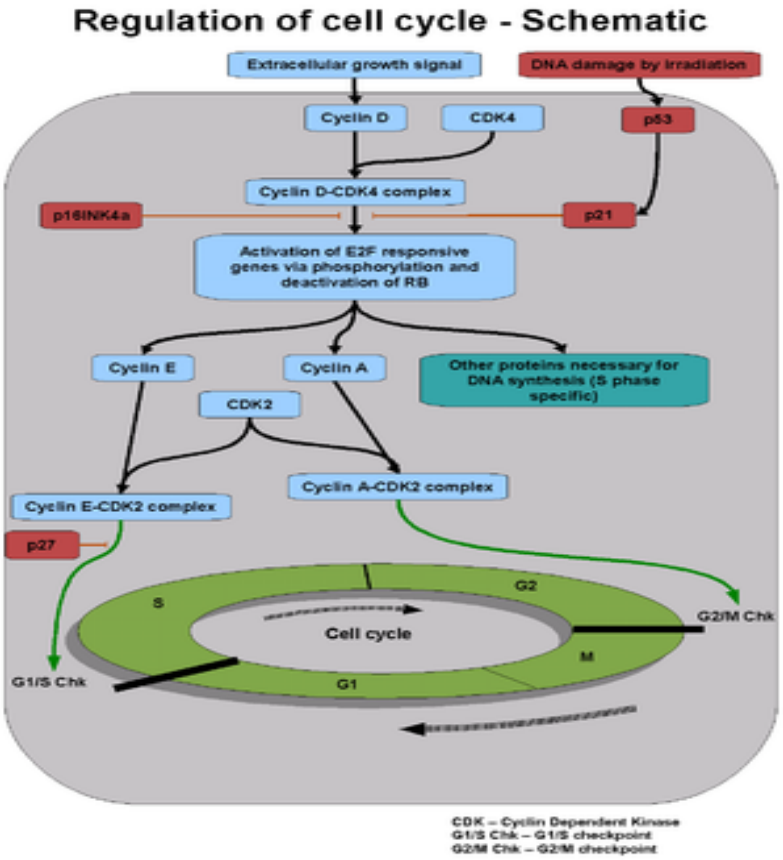


Figure No.4 -Regulation of the cell cycle involves processes crucial to the survival of a cell, including the detection and repair of genetic damage as well as the prevention of uncontrolled cell division. The molecular events that control the cell cycle are ordered and directional; that is, each process occurs in a sequential fashion and it is impossible to "reverse" the cycle.

Role of cyclins and CDKs

Two key classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), determine a cell's progress through the cell cycle.[13] Many of the genes encoding cyclins and CDKs are conserved among all eukaryotes components. Cyclins form the regulatory subunits and CDKs the catalytic subunits of an activated heterodimer.

Inhibitors:

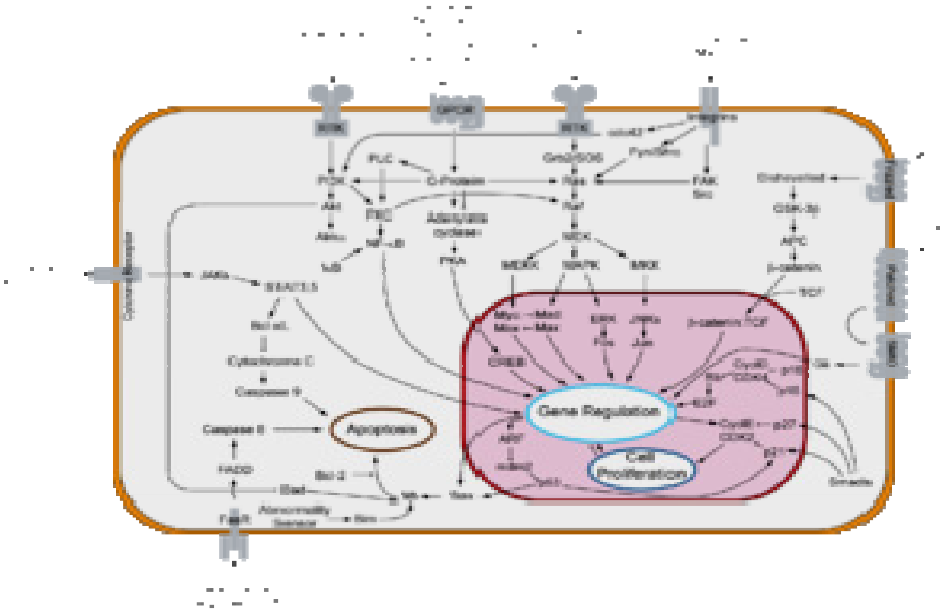


Figure No.5 -Overview of signal transduction pathways involved in apoptosis, also known as "programmed cell death".

Role in tumor formation:

A disregulation of the cell cycle components may lead to tumor formation. As mentioned above, some genes like the cell cycle inhibitors, RB, p53 etc., when they mutate, may cause the cell to multiply uncontrollably, forming a tumor. Although the duration of cell cycle in tumor cells is equal to or longer than that of normal cell cycle, the proportion of cells that are in active cell division (versus quiescent cells in G_0 phase) in tumors is much higher than that in normal tissue. Thus there is a net increase in cell number as the number of cells that die by apoptosis or senescence remains the same

MECHANISM:

Cancer is fundamentally a disease of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, genes which regulate cell growth and differentiation must be altered.[14] Genetic changes can occur at many levels, from gain or loss of entire chromosomes to a mutation affecting a single DNA nucleotide. There are two broad categories of genes which are affected by these changes.

Oncogenes may be normal genes which are expressed at inappropriately high levels, or altered genes which have novel properties. In either case, expression of these genes promotes the malignant phenotype of cancer cells. Tumor suppressor genes are genes which inhibit cell division, survival, or other properties of cancer cells. Tumor suppressor genes are often disabled by cancer-promoting genetic changes. Typically, changes in many genes are required to transform a normal cell into a cancer cell.[15]

Epigenetics

Epigenetics is the study of the regulation of gene expression through chemical, non-mutational changes in DNA structure. The theory of epigenetics in cancer pathogenesis is that non-mutational changes to DNA can lead to alterations in gene expression. Normally, oncogenes are silent, for example, because of DNA methylation. Loss of that methylation can induce the aberrant expression of oncogenes, leading to cancer pathogenesis.

Oncogenes

Oncogenes promote cell growth through a variety of ways. Many can produce hormones, a "chemical messenger" between cells which encourage mitosis, the effect of which depends on the signal transduction of the receiving tissue or cells.

The change of an oncogene from normal to cancerous function can be caused by a simple point mutation in the sequence of a gene. For example, a change in the ras oncogene, located on human chromosome 11, from guanine to cytosine is frequently associated with bladder cancer. This simple change results in glycine at amino acid #12 being substituted with a valine. This dramatically changes the function of the G-protein encoded by the ras gene. Normally, the protein cycles from an inactive to active state by change the bound GDP to GTP. The mutation does not allow the release of GTP, and the protein is continuously active. Because the signal delivered by the ras oncoprotein is continuously delivered, the cell continues to grow and divide. This unabated growth leads to the bladder cancer.

The following table summarizes the types of molecular changes that can be associated with the activation of an oncogene.

Table No.2

Molecular Change	Effect On Oncogene
Translocation	Philadelphia chromosome contains a bcr1/abl fusion that activates the abl protein kinase activity; Burkitt lymphoma result from the placement of the c-myc next to an enhancer in B lymphocytes
Point Mutations	See ras discussion above
Deletions	See v-erbB discussion above
Insertional activation	A retrovirus without an oncogene may insert near a proto-oncogene and activate the proto-oncogene by increasing its expression 30-100 fold. This activation is not immediate, but can take several months. The LTRs of retroviruses contain powerful promoters and enhancer sequences that presumably are responsible for the increased expression. The insertion can occur on either side of the proto-oncogene or in its intron. This type of activation has been associated with c-myc and c-myb.
Amplification	Amplification of the proto-oncogene has also been associated with the onset of cancer. This has been best studied in cell culture.

TUMOR MARKERS:

Tumor markers are molecules occurring in blood or tissue that are associated with cancer and whose measurement or identification is useful in patient diagnosis or clinical management. The ideal marker would be a "blood test" for cancer in which a positive result would occur only in patients with malignancy, one that would correlate with stage and response to treatment and that was easily and reproducibly measured.

Tumor markers can be used for one of four purposes:

1) Screening a healthy population or a high risk population for the presence of cancer;
2) Making a diagnosis of cancer or of a specific type of cancer;
3) Determining the prognosis in a patient;
4) Monitoring the course in a patient in remission or while receiving surgery, radiation, or chemotherapy.

Tumor Antigens

Include markers defined by both monoclonal antibodies and polyclonal antisera, often the so called oncofetal antigens. The oncofetal substances, present in embryo or fetus, diminish to low levels in the adult but reappear in the tumor.

Carcinoembryonic Antigen

Tumor marker, CEA: Carcinoembryonic antigen (CEA) is a protein found in many types of cells but associated with tumors and the developing fetus. CEA is tested in blood. The normal range is <2.5 ng/ml in an adult non-smoker and <5.0 ng/ml in a smoker.

Alpha-Fetoprotein

Alpha-Fetoprotein is a normal fetal serum protein synthesized by the liver, yolk sac, and gastrointestinal tract that shares sequence homology with albumin. It is a major component of fetal plasma, reaching a peak concentration of 3 mg/ml at 12 weeks of gestation.

CA 125

CA125 is an antigen present on 80 percent of nonmucinous ovarian carcinomas. It is defined by a monoclonal antibody (OC125) that was generated by immunizing laboratory mice with a cell line established from human ovarian carcinoma.

Prostate-Specific Antigen

The PSA screening test is a blood test that looks for a specific tumor marker. In general, tumor markers are produced by the tumor itself or by our body in response to the presence of cancer or non-cancerous conditions.

Bladder tumor antigen (BTA)

BTA is found in the urine of many patients with bladder cancer. It may be a sign of some non-cancerous conditions, too, such as kidney stones or urinary tract

infections. The results of the test are reported as either positive (BTA is present) or negative (BTA is not present).

CA 27.29

CA 27.29 is another marker that can be used to follow patients with breast cancer during or after treatment. This test measures the same marker as the CA 15-3 test.

Calcitonin

Calcitonin is a hormone produced by cells called Para follicular C cells in the thyroid gland. It normally helps regulate blood calcium levels. Normal calcitonin levels are below 5 to 12 pg/ml (pictograms per milliliter). (A pictogram is one trillionth of a gram.) In medullary thyroid carcinoma (MTC), a rare cancer that starts in the Para follicular C cells, blood levels of this hormone are often greater than 100 pg/ml.

HER2 (also known as HER2/neu, erbB-2, or EGFR2)

HER2 is a protein that tells breast cancer cells to grow. It is elevated in some breast cancers. Higher than normal levels can be found in some other cancers, too. The HER2 level is usually found by testing a sample of the cancer tissue itself, not the blood. About 1 in 5 breast cancers test positive for HER2.

Human chronic gonadotropin (HCG)

HCG (also known as beta-HCG) blood levels are elevated in patients with some types of testicular and ovarian cancers (germ cell tumors) and in gestational trophoblastic disease, mainly choriocarcinoma. They are also higher in some people with mediastinal germ cell tumors cancers in the middle of the chest (the mediastinum) that start in the same cells as germ cell tumors of the testicles and ovaries.

Immunoglobulin

Immunoglobulins are not really tumor markers but antibodies, which are blood proteins normally made by immune system cells to help fight germs. There are many types of immunoglobulin, including IgA, IgG, IgD, and IgM.

PREVENTION

Modifiable ("lifestyle") risk factors:

The vast majority of cancer risk factors are environmental or lifestyle-related in nature leading to the claim that cancer is a largely preventable disease.[16] Examples of modifiable cancer risk factors include alcohol consumption (associated with increased risk of oral, esophageal, breast, and other cancers), smoking (although 20% of women with lung cancer have never smoked, versus 10% of men[17]), physical inactivity (associated with increased risk of colon, breast, and possibly other cancers), and being overweight / obese (associated with colon, breast, endometrial, and possibly other cancers). .

Diet:

The consensus on diet and cancer is that obesity increases the risk of Proposed dietary interventions for primary cancer risk reduction generally gain support from epidemiological association studies. Examples of such studies include reports that reduced meat consumption is associated with decreased risk of colon cancer,[18] and reports that consumption of coffee is associated with a reduced risk of liver cancer.[19]

Vitamins

Epidemiological studies have shown that low vitamin D status is correlated to increased cancer risk.

Chemoprevention

The concept that medications could be used to prevent cancer is an attractive one. Daily use of tamoxifen, a selective estrogen receptor modulator (SERM), typically for 5 years, has been demonstrated to reduce the risk of developing breast

cancer in high-risk women by about 50%. A recent study reported that the selective estrogen receptor modulator raloxifene has similar benefits to tamoxifen in preventing breast cancer in high-risk women, with a more favorable side effect profile.[20]

Raloxifene is a SERM like tamoxifen; it has been shown (in the STAR trial) to reduce the risk of breast cancer in high-risk women equally as well as tamoxifen. In this trial, which studied almost 20,000 women, raloxifene had fewer side effects than tamoxifen, though it did permit more DCIS to form.

Finasteride, a 5-alpha-reductase inhibitor, has been shown to lower the risk of prostate cancer, though it seems to mostly prevent low-grade tumors.[21] The effect of COX-2 inhibitors such as rofecoxib and celecoxib upon the risk of colon polyps have been studied in familial adenomatous polyposis patients[22] and in the general population.[23, 24]

Genetic testing

Genetic testing for high-risk individuals is already available for certain cancer-related genetic mutations. Carriers of genetic mutations that increase risk for cancer incidence can undergo enhanced surveillance, chemoprevention, or risk-reducing surgery. Early identification of inherited genetic risk for cancer, along with cancer-preventing interventions such as surgery or enhanced surveillance, can be lifesaving for high-risk individuals.

Table No.3

Gene	Cancer types	Availability
BRCA1, BRCA2	Breast, ovarian, pancreatic	Commercially available for clinical specimens
MLH1, MSH2, MSH6, PMS1, PMS2	Colon, uterine, small bowel, stomach, urinary tract	Commercially available for clinical specimens

Vaccination

Prophylactic vaccines have been developed to prevent infection by oncogenic infectious agents such as viruses, and therapeutic vaccines are in development to stimulate an immune response against cancer-specific epitopes.[25] A preventive human papillomavirus vaccine exists that targets certain sexually transmitted strains of human papillomavirus that are associated with the development of cervical cancer and genital warts. The only two HPV vaccines on the market as of October 2007 are Gardasil and Cervarix. There is also a hepatitis B vaccine, which prevents infection with the hepatitis B virus, an infectious agent that can cause liver cancer. A canine melanoma vaccine has also been developed.[26]

TREATMENT:

Cancer can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods. The choice of therapy depends upon the location and grade of the tumor and the stage of the disease, as well as the general state of the patient (performance status).

Surgery

Non-hematological cancers can be cured if entirely removed by surgery but this is not always possible. When the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible. In the Halstedian model of cancer progression, tumors grow locally, then spread to the lymph nodes, then to the rest of the body. This has given rise to the popularity of local-only treatments such as surgery for small cancers. Even small localized tumors are increasingly recognized as possessing metastatic potential.

Examples of surgical procedures for cancer include mastectomy for breast cancer and prostatectomy for prostate cancer.

Radiation therapy

Radiation therapy (also called radiotherapy, X-ray therapy, or irradiation) is the use of ionizing radiation to kill cancer cells and shrink tumors. Radiation therapy can be administered externally via external beam radiotherapy (EBRT) or internally via brachytherapy. The effects of radiation therapy are localised and confined to the region being treated. Radiation therapy injures or destroys cells in the area being treated (the "target tissue") by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, stomach, uterus, or soft tissue sarcomas. Radiation is also used to treat leukemia and lymphoma.

Chemotherapy

The term "chemotherapy" usually refers to cytotoxic drugs which affect rapidly dividing cells in general, in contrast with targeted therapy . Chemotherapy drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and The treatment of some leukaemias and lymphomas requires the use of high-dose chemotherapy, and total body irradiation (TBI)

Targeted therapies

Targeted therapy, which first became available in the late 1990s, has had a significant impact in the treatment of some types of cancer, and is currently a very active research area. This constitutes the use of agents specific for the deregulated proteins of cancer cells. Small molecule targeted therapy drugs are generally inhibitors of enzymatic domains on mutated, overexpressed, or otherwise critical proteins within the cancer cell. Prominent examples are the tyrosine kinase inhibitors imatinib (Gleevec/Glivec) and gefitinib (Iressa).

Monoclonal antibody therapy is another strategy in which the therapeutic agent is an antibody which specifically binds to a protein on the surface of the cancer cells. Examples include the anti-HER2/neu antibody trastuzumab (Herceptin) used in

breast cancer, and the anti-CD20 antibody rituximab, used in a variety of B-cell malignancies.

Immunotherapy :

Cancer immunotherapy refers to a diverse set of therapeutic strategies designed to induce the patient's own immune system to fight the tumor. Contemporary methods for generating an immune response against tumours include intravesical BCG immunotherapy for superficial bladder cancer, and use of interferons and other cytokines to induce an immune response in renal cell carcinoma and melanoma patients. Vaccines to generate specific immune responses are the subject of intensive research for a number of tumours, notably malignant melanoma and renal cell carcinoma. Sipuleucel-T is a vaccine-like strategy in late clinical trials for prostate cancer in which dendritic cells from the patient are loaded with prostatic acid phosphatase peptides to induce a specific immune response against prostate-derived cells.

Hormonal therapy

The growth of some cancers can be inhibited by providing or blocking certain hormones. Common examples of hormone-sensitive tumors include certain types of breast and prostate cancers. Removing or blocking estrogen or testosterone is often an important additional treatment. In certain cancers, administration of hormone agonists, such as progestogens may be therapeutically beneficial.

Angiogenesis inhibitors

Angiogenesis inhibitors prevent the extensive growth of blood vessels (angiogenesis) that tumors require to survive. Some, such as bevacizumab, have been approved and are in clinical use.

CANCER STATISTICS [27]

Each year, the American Cancer Society estimates the number of new cancer cases and deaths expected in the United States in the current year and compiles the most recent data on cancer incidence, mortality, and survival based on incidence data from the National Cancer Institute, Centers for Disease Control and Prevention, and the North American Association of Central Cancer Registries and mortality data from the National Center for Health Statistics. Incidence and death rates are standardized by age to the 2000 United States standard million population. A total of 1,479,350 new cancer cases and 562,340 deaths from cancer are projected to occur in the United States in 2009. Overall cancer incidence rates decreased in the most recent time period in both men (1.8% per year from 2001 to 2005) and women (0.6% per year from 1998 to 2005), largely because of decreases in the three major cancer sites in men (lung, prostate, and colon and rectum [colorectum]) and in two major cancer sites in women (breast and colorectum). Overall cancer death rates decreased in men by 19.2% between 1990 and 2005, with decreases in lung (37%), prostate (24%), and colorectal (17%) cancer rates accounting for nearly 80% of the total decrease. Among women, overall cancer death rates between 1991 and 2005 decreased by 11.4%, with decreases in breast (37%) and colorectal (24%) cancer rates accounting for 60% of the total decrease. The reduction in the overall cancer death rates has resulted in the avoidance of about 650,000 deaths from cancer over the 15-year period. This report also examines cancer incidence, mortality, and survival.

Table No-4

CONTAINING CANCER DEATH BY SITE AND SEX[28]

Sr.No	Male	Death occurs	Female	Death occurs
1	Lung	95,400	Lung	62,000
2	Prostate	40,400	Breast	46,000
3	Colon & rectum	27,200	Colon & rectum	28,100
4	Pancreas	13,200	Pancreas	13,800
5	Lymphoma	12,820	Lymphoma	11,330
6	Leukaemia	11,100	Leukaemia	9,300
7	Stomach	8,800	Stomach	5,900
8	Eosaphagus	8,200	Ovary	14,500
9	Liver	7,700	Liver	14,500
10	Bladder	7,500	Brain	6,000
11	Brain	7,300	Uterus	10,700
12	Kidney	7,100	Kidney	5,000

CANCER: HERBAL MEDICINE

Extensive research during the last 30 years has revealed much about the biology of cancer. Drugs used to treat most cancers are those that can block cell signaling, including growth factor signaling (eg:,epidermal growth factor); prostaglandin production(eg:,COX-2); inflammation(eg:,inflammatory cytokines:NF-kappaB,TNF,IL-1, IL-6, chemokines); drug resistance gene products(eg:multi-drug resistance); cell cycle proteins(eg:Cyclin D1 and cyclin E); angiogenesis(eg: vascular endothelial growth factor); invasion (eg: matrix metalloproteinases); antiapoptosis (eg:bcl-2, bcl-X(L), XIAP, surviving, FLIP); and cellular proliferation (eg:c-myc, AP-1, growth factors). Numerous reports have suggested that ayurvedic plants and their components mediate their effects by modulating several of these recently identified therapeutic targets. However, ayurvedic medicine requires rediscovery in light of our current knowledge of allopathic(modern) medicine. In particular, cancer patients are reported to benefit from treatment with herbal medicine and survivability in many cases is significantly enhanced. Recent studies showed the anti-oxidative and superoxide scavenging activities of individual active components of herbal medicine for their inhibitory activities on lipid peroxidation and anti-cancer properties. Individual herbal medicines show antipyretic, analgesic and anti-inflammatory and anti-cancer effects. Numerous in vitro studies of herbal medicine on different cell lines and in vivo study of herbal medicine have been reported. However, the mechanism of actions remain unclear.

CHAPTER - II

Review of literature

REVIEW OF LITERATURE

Kalita S, et al., (2014) has studied the Chlorambucil and ascorbic acid-mediated anticancer activity and hematological toxicity in Dalton's ascites lymphoma-bearing mice. Based on these study combination treatment of mice with ascorbic acid plus chlorambucil showed less histopathological changes in kidney as compared to chlorambucil treatment alone, thus, ascorbic acid is effective in reducing chlorambucil-induced renal toxicity in the hosts.[29]

Martha KR, et al., (2013) has studied the Cisplatin and dietary ascorbic acid mediated changes in the mitochondria of dalton's lymphoma bearing mice.Combination treatment increase the antitumor activity and decrease the cisplatin induced toxicity in the host.[30]

Surya bali Prasad, et al., (2010) has studied the Cyclophosphamide and ascorbic acid-mediated ultrastructural and biochemical changes in Dalton's lymphoma cells in vivo. The observed ascorbic acid plus cyclophosphamide-mediated decrease in reduced glutathione (GSH) in tumor cells may play an important role in the antitumor activity of cyclophosphamide by weakening cellular antioxidant-mediated defense mechanism, thereby increasing tumor cell's susceptibility to cell death. **[31]**

Nicol BM et al., (2002) has studied the Sialic acid changes in Dalton's lymphoma-bearing mice after cyclophosphamide and cisplatin treatment. The study showed that significant decrease in the sialic acid content of Dalton's lymphoma cells after cisplatin or cyclophosphamide treatment may bring about specific changes in tumour cells which could be associated with tumour regression. [32]

Osama A.Badari, et al., (1999) studied the Thymoquinone attenuates ifosfamide-induced Fanconi syndrome in rats and enhances its antitumor activity in mice.These observations demonstrate that TQ may improve the therapeutic efficacy of IFO by decreasing IFO-induced nephrotoxicity and improving its antitumor activity. [33]

Kirsten Börner, et al., (2000) studied the Metabolism of Ifosfamide to Chloroacetaldehyde Contributes to Antitumor Activity In Vivo. On a molar basis

chloroacetaldehyde was seven times less potent as 4-hydroxyifosfamide.These study showed that the basis of achieved AUC values, chloroacetaldehyde seems to exhibit a similar antitumor activity to 4-hydroxyifosfamide. [34]

C Bokemeyer, et al., (1996) has studied the Silibinin protects against cisplatin-induced nephrotoxicity without compromising cisplatin or ifosfamide anti-tumour activity. he current data form the basis for a clinical study using cisplatin-based combination chemotherapy including silibinin, in patients with testicular cancer in order to reduce the acute and long- term nephrotoxic potential of cisplatin. **[35]**

M Markman, et al., (1992) has studied the Ifosfamide and mesna in previously treated advanced epithelial ovarian cancer: activity in platinum-resistant disease.These study showed that Single-agent ifosfamide has modest but unequivocal activity in platinum-resistant ovarian cancer. Further studies of this drug used as a front-line agent along with an organoplatinum compound or as part of a dose-intensification program with bone marrow, peripheral stem cell, or colony-stimulating factor support are indicated. In addition, single-agent ifosfamide is a reasonable standard second-line treatment strategy in appropriately selected patients with platinum-refractory ovarian cancer. [36]

Kuśnierczyk H, et al., **(1986)** has studied the Antitumor Activity of Optical Isomers of Cyclophosphamide, Ifosfamide and Trofosfamide as Compared to Clinically Used Racemate+. These study shown that the stereodifferentiation of anti-tumor effect of enantiomers was not outstanding although quite consistently in favor of levorotatory forms. [37]

W. Brade,et al., (1986) has studied the Comparative activity of ifosfamide and cyclophosphamide.More comparative controlled clinical trials are needed in ovarian cancer, breast cancer, malignant lymphomas, sarcomas and cervical cancer, in which IFO has already shown sufficient single-agent activity.These study showed that to its lower level of cross-resistance with a variety of heterocyclic products, but also with other alkylating agents, in addition to its use in induction chemotherapy, IFO is an important second-line agent in many clinical situations. [38]

Muthu Irulappan Sriram, et al., (2010) has studied the Antitumor activity of silver nanoparticles in Dalton's lymphoma ascites tumor model. These findings confirm the antitumor properties of AgNPs, and suggest that they may be a cost-effective alternative in the treatment of cancer and angiogenesis-related disorders. [39]

Meghna R Adhvaryu, et al., (2008) has studied the Anti-tumor activity of four Ayurvedic herbs in Dalton lymphoma ascites bearing mice and their short-term in vitro cytotoxicity on DLA-cell-line.The study showed All the four herbs showed in vitro cytotoxic activity against DLA cell-line. Moreover inter group comparison of all the four herbs for anti-tumor activity showed efficacy in the following order--T. cordifolia > Z. mauritiana > or = C. longa > O. sanctum respectively. [40]

Benjamin RS, et al.,(1993) has studied the Single-agent ifosfamide studies in sarcomas of soft tissue and bone: the M.D. Anderson experience.These study showed that Ifosfamide has demonstrated clinically useful antitumor activity in our hands against most sarcoma subtypes. Our studies suggest a dose-response relationship for ifosfamide. At a total dose of 6 g/m2 per course, the overall response rate was 10%; at 10 g/m2 per course, it rose to 21%. Future clinical trials will determine ifosfamide's role in combination chemotherapy and more clearly define the best schedule or schedules for the uroprotective administration of mesna. [41]

H. Glazman-kuśnierczyk, et al., (1992) has studied the Antitumor Activity Evaluation of Bromine-Substituted Analogues of Ifosfamide. I. Stereodifferentiaton of Biological Effects Amd Selection of the Most Potent Compounds.These study showed that the effect four compounds which have been shown therapeutically more effective than both referential drugs, were selected for further evaluation in mice bearing advanced tumours. [42]

Vanhoefer U, et al.,(2000) has studied the Ifosfamide-based drug combinations, preclinical evaluation of drug interactions and translation into the clinic.These study showed that the data reported here demonstrate that in vitro ifosfamide may potentiate

the antitumor activity of a variety of cytotoxic agents and therefore merits further clinical evaluation in drug combinations (eg, taxanes, anthracyclines). [43]

B Samuel Thavamani, et al.,(2014) has studied the Anticancer activity of *cissampelos pareira* against dalton's lymphoma ascites bearing mice.These study showed that *C. pariera* exhibited significant *in vitro* and *in vivo* anti-tumor activities and that it was reasonably imputable to its increasing endogenous mechanism of antioxidant property. [44]

Shivakumar. B.S, et al., has studied the Evaluation of In -Vitro Anticancer Activity of Barlaria buxifolia Linn Extracts Against Dalton's Lymphoma Ascites Cell line .The study showed that the present study suggested that the methanolic extract of root from Barlaria buxifolia is cytotoxic to DLA cell lines. The presence of flavonoids and related phytoconstituents may be responsible for the activity. Further studies warranted, for isolation of the constituents responsible for the activity and also to explore the exact mechanism of action of the activity. [45]

Purushoth Prabhu, et al., has studied that the Invitro and Invivo anticancer activity of Ethanolic extract of Canthium Parviflorum Lam on DLA and Hela cell lines. These study showed that Anticancer activity of Canthium Parviflorum was may be due to flavonoid present in the plant. Further studies are also in process to evaluate the most potent fraction of the plant and to isolate the constituents of the fractions. [46]

Christian M. Kurbacher, et al., (1996) has studied that Ascorbic acid (vitamin C) improves the antineoplastic activity of doxorubicin, cisplatin, and paclitaxel in human breast carcinoma cells in vitro. Combination effects between Vit C and DDP or Tx were partly synergistic and partly additive or sub additive whereas a consistent synergism was found between Vit C and DOX. [47]

Perrone G, et al.,(2009) Ascorbic acid inhibits antitumor activity of bortezomib in vivo. Therefore, our results for the first time show that vitamin C can significantly reduce the activity of bortezomib treatment in vivo; and importantly, suggest that

patients receiving treatment with bortezomib should avoid taking vitamin C dietary supplements. [48]

Yukitoshi Takemuraa, et al.,(2010) has studied the High dose of ascorbic acid induces cell death in mesothelioma cells. This study shows that ascorbic acid may have benefits for patients with mesothelioma. **[49]**

Anirudha giri, et al.,(1998) has studied the Vitamin C mediated protection on cisplatin induced mutagenicity in mice. These study shows combined treated hosts the frequency of all the mutagenic parameters were always significantly less than that treated with cisplatin alone. These findings suggest a protective role of ascorbic acid against cisplatin induced mutagenic potentials. **[50]**

Steve Harakeh, et al.,(2006) has studied the Ascorbic Acid Induces Apoptosis in Adult T-cell Leukemia. These study shows that Ascorbic acid was found to reduce the proliferation of cells and induce apoptosis by the modulation of p53, p21, Bcl-2 and Bax. **[51]**

Ewan Cameron, et al., (1979) has studied the Ascorbic acid and cancer review. This study showed that there is evidence from both human and experimental animal studies that the development and progress of cancer evokes an increased requirement for ascorbic acid. [52]

Aristides Polyzos, et al., (2009) has studied the Cisplatin–Ifosfamide–Gemcitabine as Salvage Chemotherapy in Ovarian Cancer Patients Pretreated with Platinum Compounds and Paclitaxel. These study shows that the efficacy of this three-drug combination in patients with platinum-resistant tumors was moderate. **[53]**

CHAPTER - III

Research envisaged

FOCUS OF THE PRESENT STUDY

The Ifosfamide induced major side effects include myelosuppression, pancytopenia, anaemia, thrombocytopaenia and/or leukopaenia, renal toxicity, gastrointestinal toxicity, fertility disorders and neurotoxcity. Hepatotoxicity and pneumotoxicity have been reported only infrequently. In the endeavor to decrease drug induced toxicity in the host without decreasing the therapeutic efficacy, the use of anticancer drugs such as cisplatin, cyclophosphamide, paclitaxel and arsenic trioxide in combination with vitamin C (2-Oxo-L-threo-hexono-1,4-lactone-2,3-enediol.

The significance of vitamin C in relation to cancer chemotherapy and possibility of development of Ifosfamide induced toxicity in mice, the present study has been undertaken to assess the modulatory effect of dietary ascorbic acid on Ifosfamide mediated antitumor efficacy and haematological and renal toxicity in mice.

PLAN OF WORK

Anticancer activity of Ifosfamide and ascorbic acid mediated haemotological toxicity in Dalton's ascites Lymphoma bearing mice.

It is planned to carryout this work as outlined below.

1. In-vivo evaluation of anticancer activity.
    a. Induction of cancer using DLA cell lines
    b. Treatment protocol.
    c. Study of various biochemical parameters in the blood and Derived parameters.
    d. Histopathological parameters.

BIOCHEMICAL PARAMETERS

- Hematological Parameters
  - WBC Count
  - RBC Count
  - Platelet Count
  - Hemoglobin content
  - Packed cell Volume

- Serum Enzyme and Lipid Profile
  - Aspartate amino Transferase (AST)
  - Alanine amino Transferase (ALT)
  - Alkaline Phosphatase (ALP)
  - Total Cholesterol (TC)
  - Triglyceride (TG)

DERIVED PARAMETERS

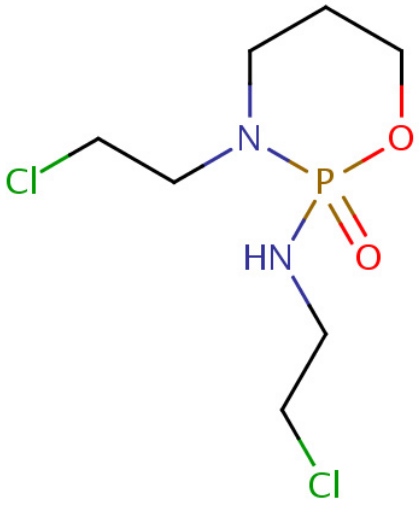
- Body weight
- Percentage increase in Life span
- Cancer cell count

CHAPTER – IV

Drug profile

IFOSFAMIDE

Ifosfamide (also marketed as Ifex) is a nitrogen mustard alkylating agent used in the treatment of cancer.[54] It is sometimes abbreviated as "IFO".[55]



SYNONYM

3-(2-Chloroethyl)-2-((2-chloroethyl)amino)tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide

USES

It is given as a treatment for a variety of cancers, including:

- Testicular cancer
- Breast cancer
- Lymphoma (Hodgkin and nonHodgkin)
- Soft tis sue sarcoma
- Osteos arcoma or bone tumor
- Lung cancer
- Cervical cancer
- Ovarian cancer

ADMINISTRATION

It is a white powder which, when prepared for use in chemotherapy, becomes a clear, colorless fluid. The delivery is intravenous. Ifosfamide is often used in

conjunction with mesna to avoid internal bleeding in the patient, in particular hemorrhagic cystitis. Ifosfamide is given quickly, and in some cases can be given as quickly as an hour.

DRUG MECHANISM

The exact mechanism of ifosfamide has not been determined, but appears to be similar to other alkylating agents. Ifosfamide requires biotransformation in the liver by mixed-function oxidases (cytochrome P450 system) before it becomes active. After metabolic activation, active metabolites of ifosfamide alkylate or bind with many intracellular molecular structures, including nucleic acids. The cytotoxic action is primarily through the alkylation of DNA, done by attaching the N-7 position of guanine to its reactive electrophilic groups. The formation of inter and intra strand cross-links in the DNA results in cell death.

VOLUME OF DISTRIBUTION

Ifosfamide volume of distribution (Vd) approximates the total body water volume, suggesting that distribution takes place with minimal tissue binding. Following intravenous administration of 1.5 g/m2 over 0.5 hour once daily for 5 days to 15 patients with neoplastic disease, the median Vd of ifosfamide was 0.64 L/kg on Day 1 and 0.72 L/kg on Day 5. When given to pediatric patients, the volume of distribution was 21±1.6 L/m^2.

PROTEIN BINDING

Ifosfamide shows little plasma protein binding.

METABOLISM

Primarily hepatic. Ifosfamide is metabolized through two metabolic pathways: ring oxidation ("activation") to form the active metabolite, 4-hydroxy-ifosfamide and side-chain oxidation to form the inactive metabolites, 3-dechloro-ethylifosfamide or 2-dechloroethylifosfamide with liberation of the toxic metabolite, chloroacetaldehyde. Small quantities (nmol/mL) of ifosfamide mustard and 4-hydroxyifosfamide are detectable in human plasma. Metabolism of ifosfamide is required for the generation

of the biologically active species and while metabolism is extensive, it is also quite variable among patients.

ROUTE OF ELIMINATION

Ifosfamide is extensively metabolized in humans and the metabolic pathways appear to be saturated at high doses. After administration of doses of 5 g/m2 of 14C-labeled Ifosfamide, from 70% to 86% of the dosed radioactivity was recovered in the urine, with about 61% of the dose excreted as parent compound. At doses of 1.6–2.4 g/m2 only 12% to 18% of the dose was excreted in the urine as unchanged drug within 72 hours.

HALF LIFE

7-15 hours. The elimination half-life increase appeared to be related to the increase in Ifosfamide volume of distribution with age.

TOXICITY

LD_{50} (mouse) = 390-1005 mg/kg, LD_{50} (rat) = 150-190 mg/kg. Side effects include nausea, vomiting and myelosuppression. Toxic effects include central nervous system toxicity (confusion, hallucinations) and urotoxic effects (cystitis, blood in urine).

SIDE EFFECTS

More common

- Agitation
- black, tarry stools
- blood in the urine
- chest pain
- confusion
- cough or hoarseness
- fever or chills

- frequent urination
- hallucinations (seeing, hearing, or feeling things that are not there)
- lower back or side pain
- painful or difficult urination
- pale skin
- shortness of breath
- sore throat
- sores, ulcers, or white spots on the lips or in the mouth
- swollen glands
- troubled breathing with exertion
- unusual bleeding or bruising
- unusual tiredness or weakness

Less common

- Abdominal or stomach pain or tenderness
- bleeding gums
- bluish color
- changes in skin color
- clay colored stools
- dark urine
- decreased appetite
- dizziness
- headache
- itching
- loss of appetite

- nausea and vomiting
- pain
- pinpoint red spots on the skin
- skin rash
- swelling of the feet or lower legs
- yellow eyes or skin

Rare

- Blurred vision
- burning, numbness, tingling, or painful sensations
- confusion
- convulsions (seizures)
- dizziness, faintness, or lightheadedness when getting up suddenly from a lying or sitting position
- fast or irregular heartbeat
- sweating
- troubled breathing
- unsteadiness or awkwardness
- weakness in the arms, hands, legs or feet

CHAPTER –V

Experimental Design

EXPERIMENTAL MODEL [56]

For the study of anticancer activity, an experimental model is selected in such way that it would satisfy the following condition;

- The animal should develop cancer rapidly and reproducibly.
- Pathological changes in the site of induction should result from cancer formation.
- The symptoms should be ameliorated or prevented by a drug treatment effective in human beings.
- The drug tested should be administered orally.
- Drug dosage should approximate the optimum therapeutic range for human, scaled the test animal weight.

Selection Grouping and Acclimatization of Laboratory Animal [57]

Male Swiss albino mice (20-25 gm) were produced from animal experimental laboratory, and used throughout the study. They were housed in micro nylon boxes in a control environment (temp 25±2°C) and 12 hrs dark /light cycle with standard laboratory diet and water *ad libitum.* The study was conducted after obtaining institutional animal ethical committee clearance. As per the standard practice, the mice were segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They were fed on healthy diet and maintained in hygienic environment in our animal house.

Technique for Inducing Tumor

Various technique for induction of cancer in animals, viz, chemically induced (using DMBA/croton oil, etc) [58] virus induced, cell line induced (sarcoma – 180, ULCA fibro sarcoma and Jensen sarcoma, mouse lung fibroblast cells L-929, Dalton's Lymphoma Ascites (DLA), Ehrlich Ascites Carcinoma (EAC) [59,60,61] methods have been used in experimental studies of anticancer activity.

In the present study, cell lines induced cancer in mice was used to evaluate the anticancer activity.

EVALUATION OF ANTICANCER ACTIVITY

Induction of cancer using DLA cells

Dalton's Lymphoma Ascites (DLA) cells were supplied by Amala Cancer Research Center, Trissur, Kerala, India. The cells maintained in vivo in Swiss albino mice by intraperitoneally transplantation. While transforming the tumor cells to the grouped animal the DLA cells were aspirated from peritoneal cavity of the mice using saline. The cell counts were done and further dilution were made so that total cell should be 1 x 10^6 , this dilution was given intraperitoneally. Let the tumor grow in the mice for minimum seven days before starting treatments.

Treatment Protocol

Swiss Albino mice were divided in to five group of six each. All the animals in four groups were injected with DLA cells (1 x 10^6 cells per mouse) intraperitoneally, and the remaining one group is normal control group.

Group 1 served as the normal control.

Group 2 served as the tumor control. Group 1 and 2 receives normal diet and Water.

Group 3 served as the Treatment control, was treated with injection Ifosfamide at 10 mg/kg body weight, Intraperitoneally. [62]

Group 4 served as the treatment control, which was treated with Ascorbic acid at 10 mg/kg body weight, through Intraperitoneally.

Group 5 served as treatment control, which was treated with Ifosfamide + Ascorbic acid at 10 mg/kg body weight each, through Intraperitoneally.

CHAPTER –VI

Pharmacological evaluation

METHODOLOGY

Sample collection

In this study, drug treatment was given after the 24 hrs of inoculation, once daily for 14 days.

On day 14, after the last dose, all mice from each group were sacrificed; the blood was withdrawn from each mice by retro orbital plexus method and the following parameters were checked.

1. Hematological parameters
    a. WBC count
    b. RBC count
    c. Hb content
    d. Platelet count
    e. Packed cell volume
2. Serum enzyme and lipid profile
    a. Total Cholesterol (TC)
    b. Triglycerides (TG)
    c. Aspartate amino Transferase (AST)
    d. Alanine amino Transferase (ALT)
    e. Alkaline Phosphatase (ALP)
3. Derived parameter
    f. Body weight
    g. Life span (%)
    h. Cancer Cell Count

EVALUATION OF CLINICAL PARAMETERS [63-66]

1) Cancer cell count[67]

The fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8 ml of ice cold Normal saline or sterile Phosphate Buffer Solution and 0.1 ml of tryphan blue (0.1 mg/ml) and total numbers of the living cells were counted using heamocytometer.

$$\text{Cell count} = \frac{\text{No of cells Dilution}}{\text{Area} \times \text{Thickness of liquid film}}$$

2) Hematological parameters

i) WBC count
ii) RBC count
iii) Platelet count
iv) Hemoglobin
v) Packed Cell Volume

i) WBC count

The total WBC count was found to be increased in cancer control, when compared with normal and treated tumor-bearing mice. The total WBC count were found to decrease significantly in animals treated with plant extract when compared with cancer control, indicating that the antitumor nature of the extract.[68]

ii) RBC and Hb

RBC and Hb content decreases with tumor bearing mice when compared with Normal control mice.

iii) Platelets

In Hodgkin lymphoma, increased in platelet count often reported in laboratory finding. Hence, I investigated this parameter in the study. [69]

iv) Packed cell volume

In any case of anemia the packed cell volume is decreases.

SERUM ENZYME AND LIPID PROFILE

The serum was analysed for the following parameters

(a) Aspartate amino Transferase (AST)
(b) Alanine amino Transferase (ALT)
(c) Alkaline Phosphatase (ALP)
(d) Total Cholesterol (TC)
(e) Triglyceride (TG)

1. TOTAL CHOLESTEROL AND TRIGLYCERIDE (lipid profile)

Abnormal blood lipid profile has been associated with cancer. In Hodgkin lymphoma, high cholesterol level and low triglyceride level has been reported. Hence I investigated this parameter in the study. [70]

2. LIVER ENZYMES (AST, ALT, ALP).

Abnormal liver function seen in patient with Hodgkin lymphoma, [49] that these liver enzyme levels markedly increase in tumor bearing mice. ALP is an enzyme mainly derived from the liver, bones and in lesser amount from intestines, placenta, kidneys and leukocytes. An increase in ALP levels in the serum is frequently associated with the variety of disease. [71] ALP comprises a group of enzyme that catalyzes the phosphate esters in an alkaline environment, generating an organic radical and inorganic phosphate.

Markedly elevated serum ALP, hyperalakline-phosphatasemia, is seen predominantly with more specific disorders; including malignant biliary cirrhosis, hepatic lymphoma and sarcoidosis. [72] Hence, I investigated this parameter in this study.

DERIVED PARAMETERS [56]

1. Body weight:

All the mice were weighed, from the beginning to 15th day of the study. Average increase in body weight on the 15th day was determined.

2. Percentage increase in life span (ILS) [68]

% ILS was calculated by the following formulae

$$\%ILS = \frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \times 100$$

- All biochemical investigations were done by using COBAS MIRA PLUS-S Auto analyzer from Roche Switzerland.
- Hematological test are carried out in COBAS MICROS OT 18 from Roche.
- Newly added Hi-Tech instruments MAX MAT used for an auto analyzer for all biochemistry investigations in blood sample.

Effect of Ifosfamide and ascorbic acid on Survival Time

Animals in the first batch were divided into five groups of six animals each. Except the normal control group, the remaining groups were inoculated with DLA cells ($1x10^6$ cells/mouse) intraperitoneally on day '0' and treatment with Ifosfamide and ascorbic acid started 24 hrs after inoculation, at a dose of 10 mg/kg/day each through I.P. The normal and tumor control group was treated with same volume of 0.9% sodium chloride solution. All the treatments were given for fourteen days. The increase in life span (ILS) of each group, consisting of 6 mice was noted.

The antitumor efficacy of Ifosfamide and ascorbic acid was compared with combination of that of Ifosfamide and ascorbic acid 10 mg/kg/day each, *i.p,* for 14 days). The ILS of the treated groups was compared with that of the control group using the following calculation:

Increase in lifespan = [(T - C) / C] x 100

Where T = number of days the treated animal survived.

C = number of days control animals survived.

CHAPTER – VII

Results

Table No. 5

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON HEMATOLOGICAL PARAMETERS

TREATMENT	Total WBC Cells /mlx10^3	Rbc Count Mill/cumm	Hb Gm/dl	PCV %	Platelets Lakhs/cumm
G1	11.80 ±3.60	5.30±0.86	12.65 ±1.30	15.46±2.45	5.80±0.90
G2	13.50 ±3.60^{a**}	4.80±0.20^{a**}	9.54 ±0.92^{a**}	26.40±3.25^{a**}	4.75±0.62^{a**}
G3	14.20 ±2.75^{b**}	3.90±0.78^{b**}	10.60±1.45^{b**}	30.40±1.50^{b**}	4.50±0.96^{b**}
G4	12.42 ±2.90^{b**}	5.15±0.58^{b**}	12.50±1.32^{b**}	16.40±1.70^{b**}	5.10 ±0.80^{b**}
G5	13.20±2.75^{b**}	4.73±0.50^{b**}	11.53±1.06^{b**}	22.26±1.85^{b**}	5.30±0.92^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Treatment control (ifosfamide),

G_4 – Treatment control (ascorbic acid), G5 – Treatment control (ifosfamide + ascorbic acid)

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

.

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON HEMATOLOGICAL PARAMETERS

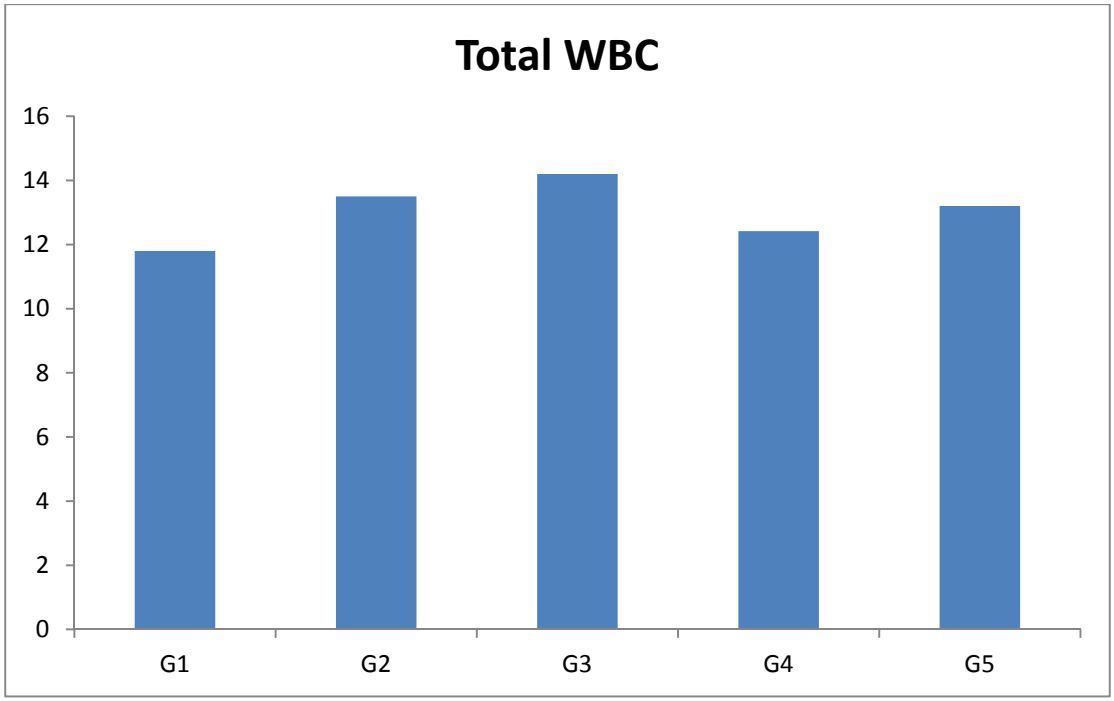


Figure No. 6

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON HEMATOLOGICAL PARAMETERS

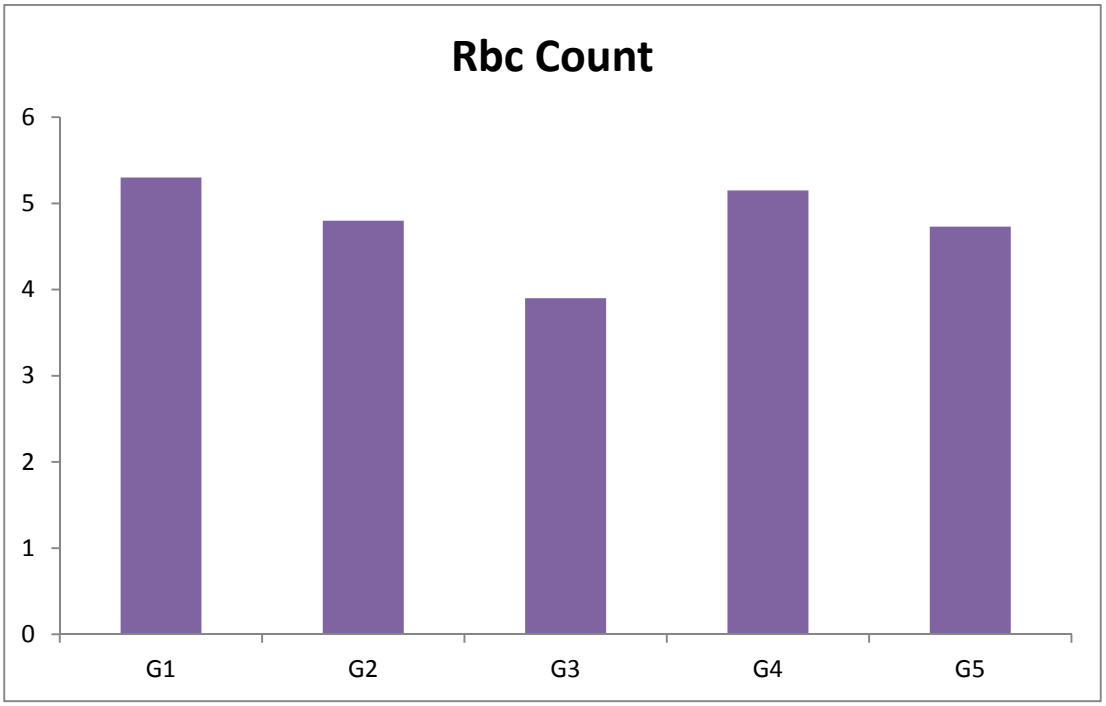


Figure No. 7

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON HEMATOLOGICAL PARAMETERS

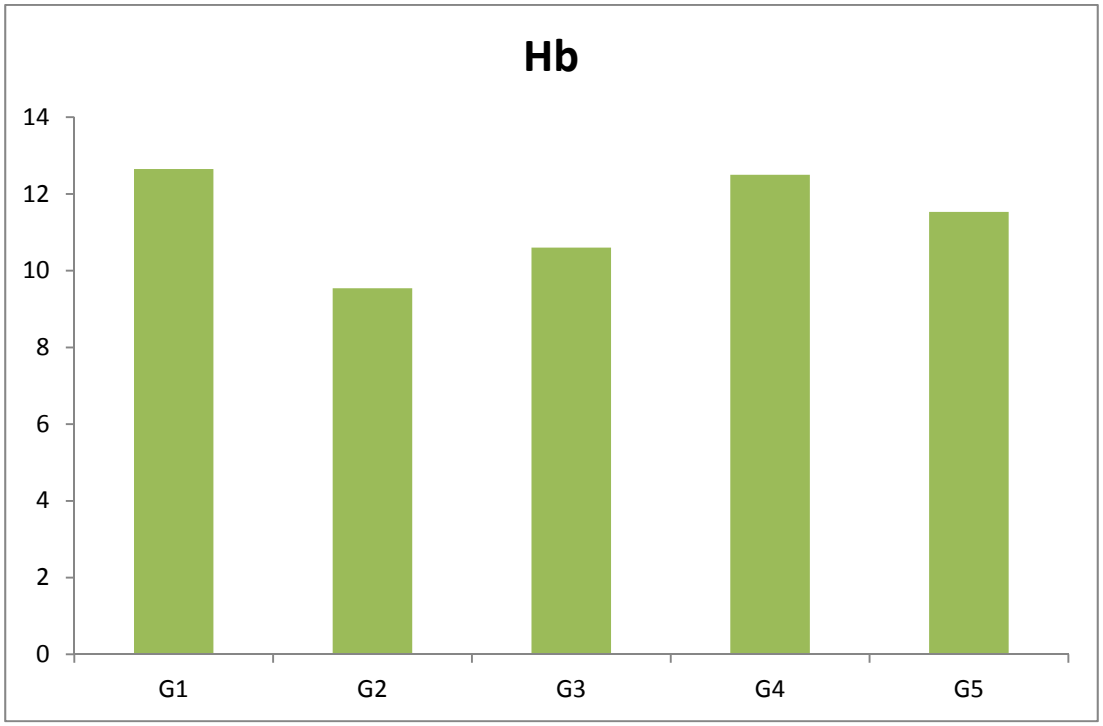


Figure No. 8

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON HEMATOLOGICAL PARAMETERS

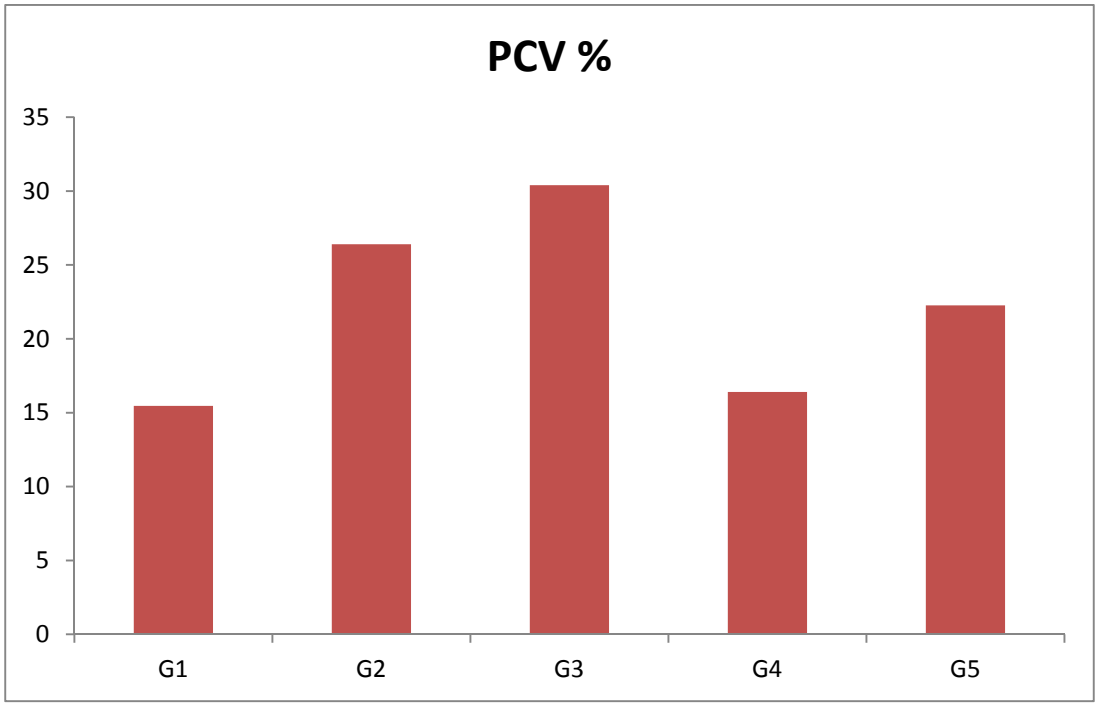


Figure No. 9

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON HEMATOLOGICAL PARAMETERS

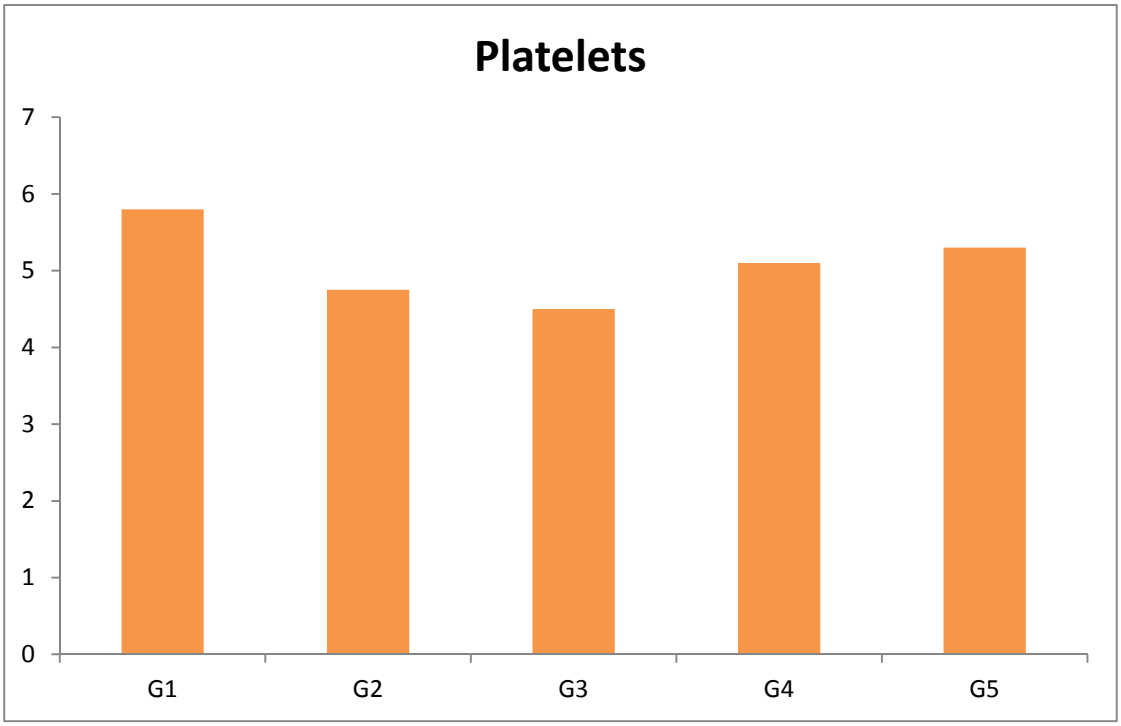


Figure No. 10

Table No.6

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON THE LIFE SPAN, BODY WEIGHT AND CANCER CELL COUNT OF TUMOR INDUCED MICE

Treatment	Number of animals	% ILS Life span	Increase in Body weight grams	Cancer cell count ml X 10^6
G_1	6	>>31 days	2.70±0.62	-
G_2	6	48%	6.80±0.96^{a**}	4.70±0.40^{a**}
G_3	6	78%	4.85±0.70^{b**}	2.59±0.22^{b**}
G_4	6	84%	3.55±0.88^{b**}	2.10±0.30^{b**}
G_5	6	80%	5.10±0.86^{b**}	2.30±0.25^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Treatment control (Ifosfamide),

G_4 – Treatment control (ascorbic acid), G5 – Treatment control (Ifosfamide+ascorbic acid)

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON THE LIFE SPAN, BODY WEIGHT AND CANCER CELL COUNT OF TUMOR INDUCED MICE

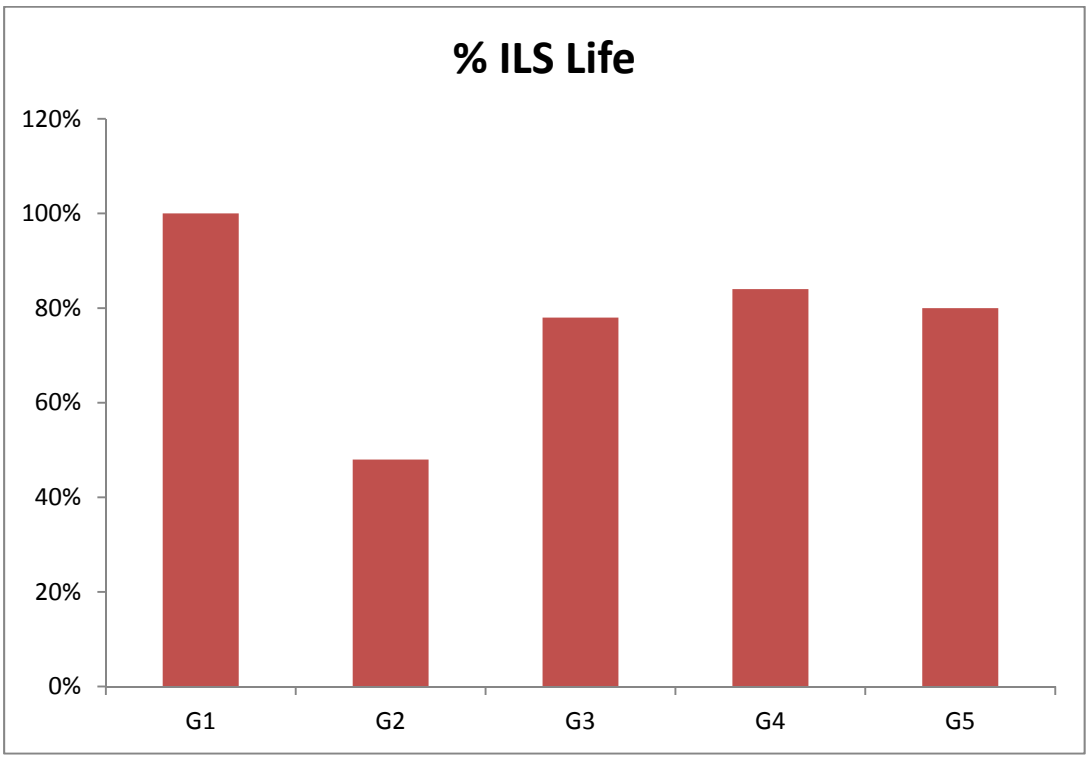


Figure No. 11

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON THE LIFE SPAN, BODY WEIGHT AND CANCER CELL COUNT OF TUMOR INDUCED MICE

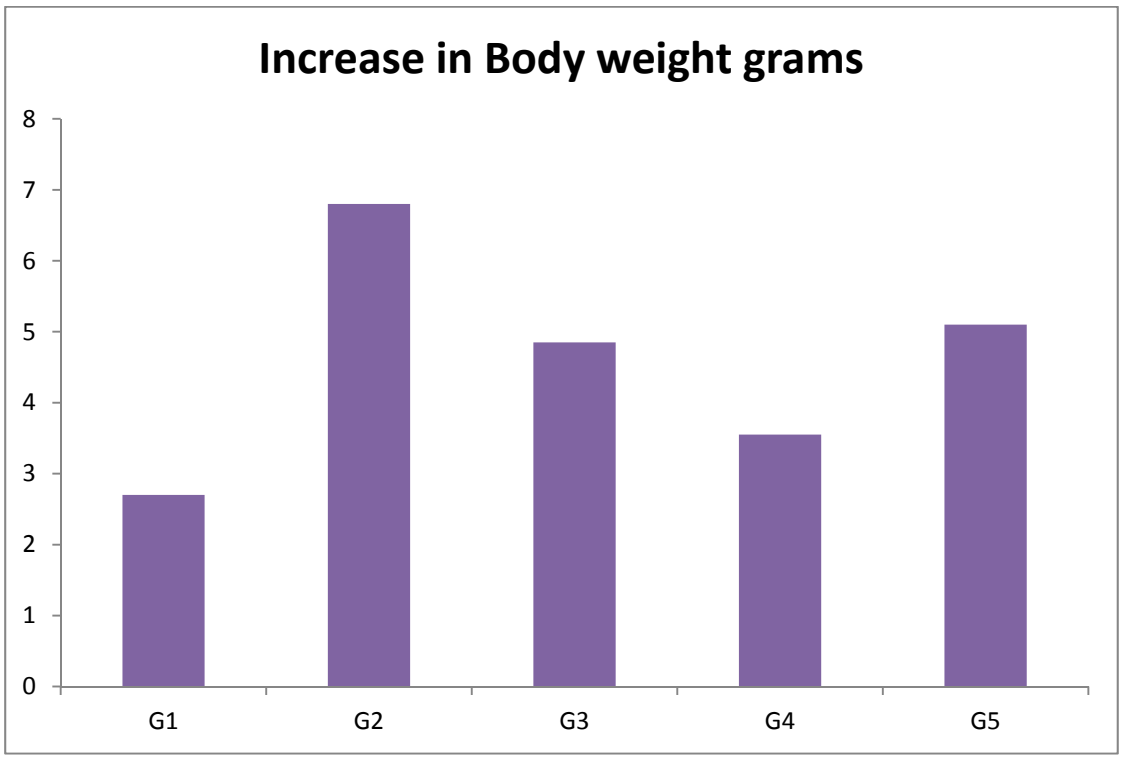


Figure No. 12

EFFECT OF IFOSFAMIDE AND ASCORBIC ACID ON THE LIFE SPAN, BODY WEIGHT AND CANCER CELL COUNT OF TUMOR INDUCED MICE

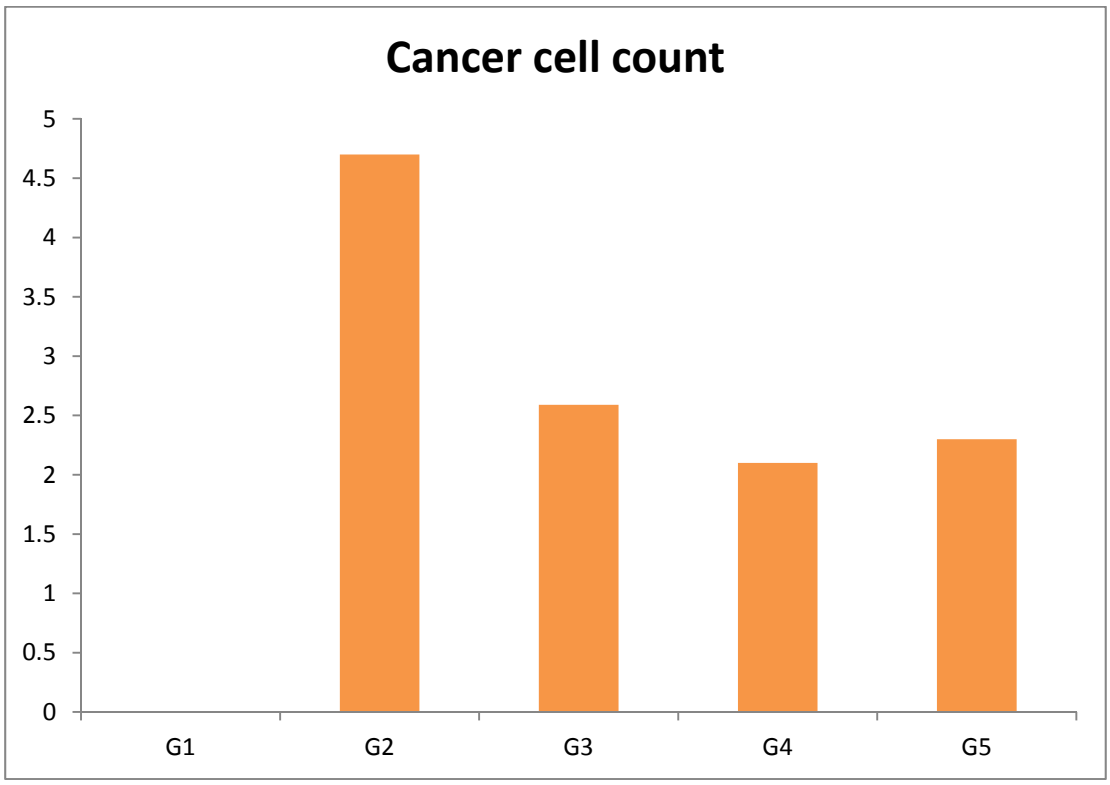


Figure No. 13

HISTOPATHOLOGICAL STUDIES

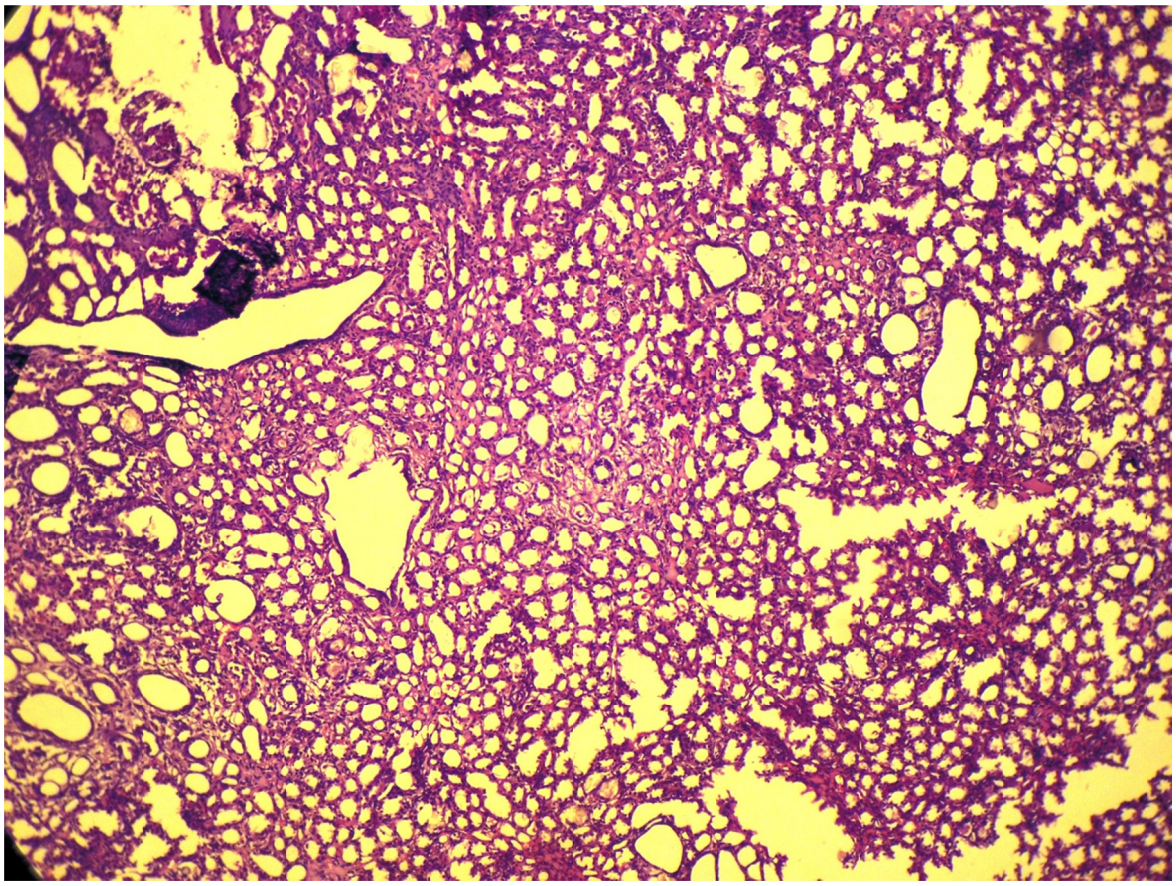


Figure. No: 14

Kidney Section of GP_1 (Normal Control) Mice

Kidney section showed normal renal architecture of both outer cortex and inner medulla

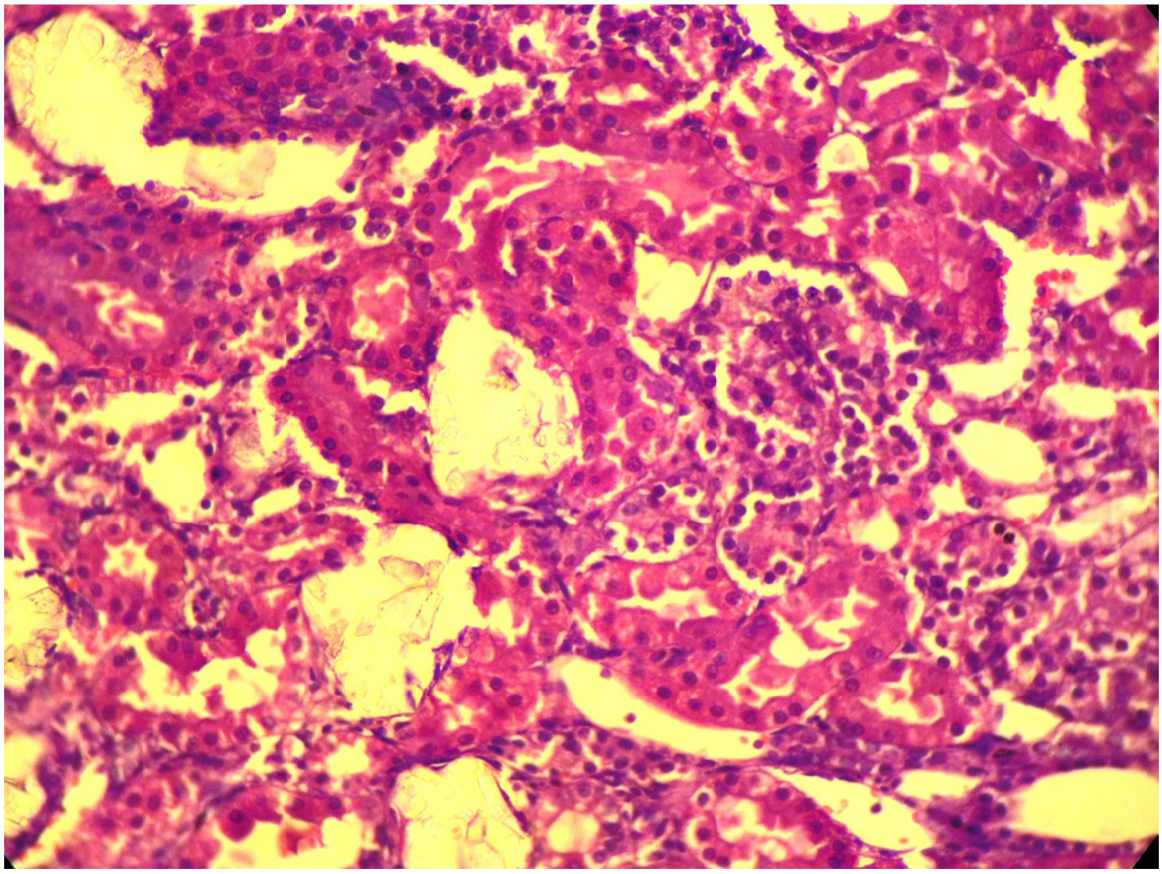


Figure. No: 15

Kidney Section of GP_2 (Toxic control) Mice

Kidney section showed many areas of tubular damages and interstitial mononuclear cell infiltration

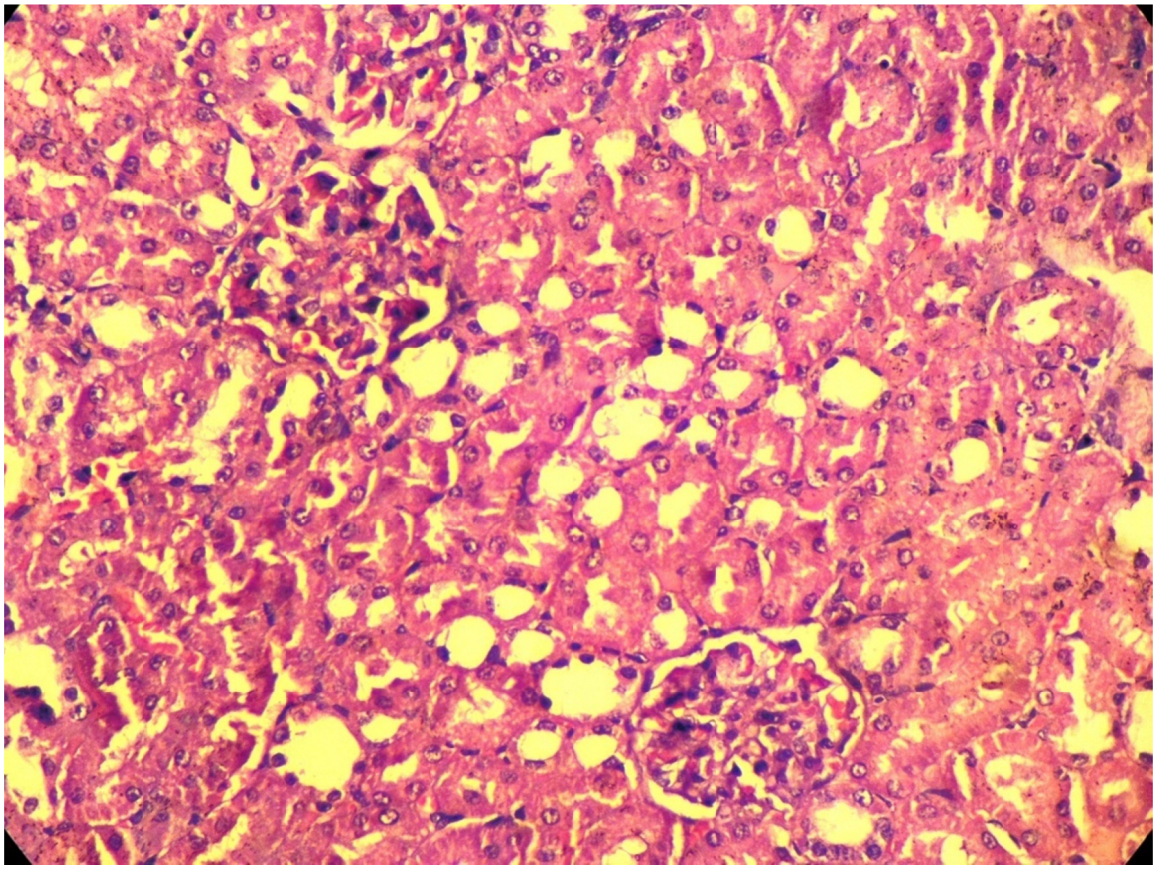


Figure. No: 16

Kidney Section of GP_3(Ifosfamide 10mg/kg/mice)

Kidney section showed many areas of tubular damages and interstitial mononuclear cell infiltration

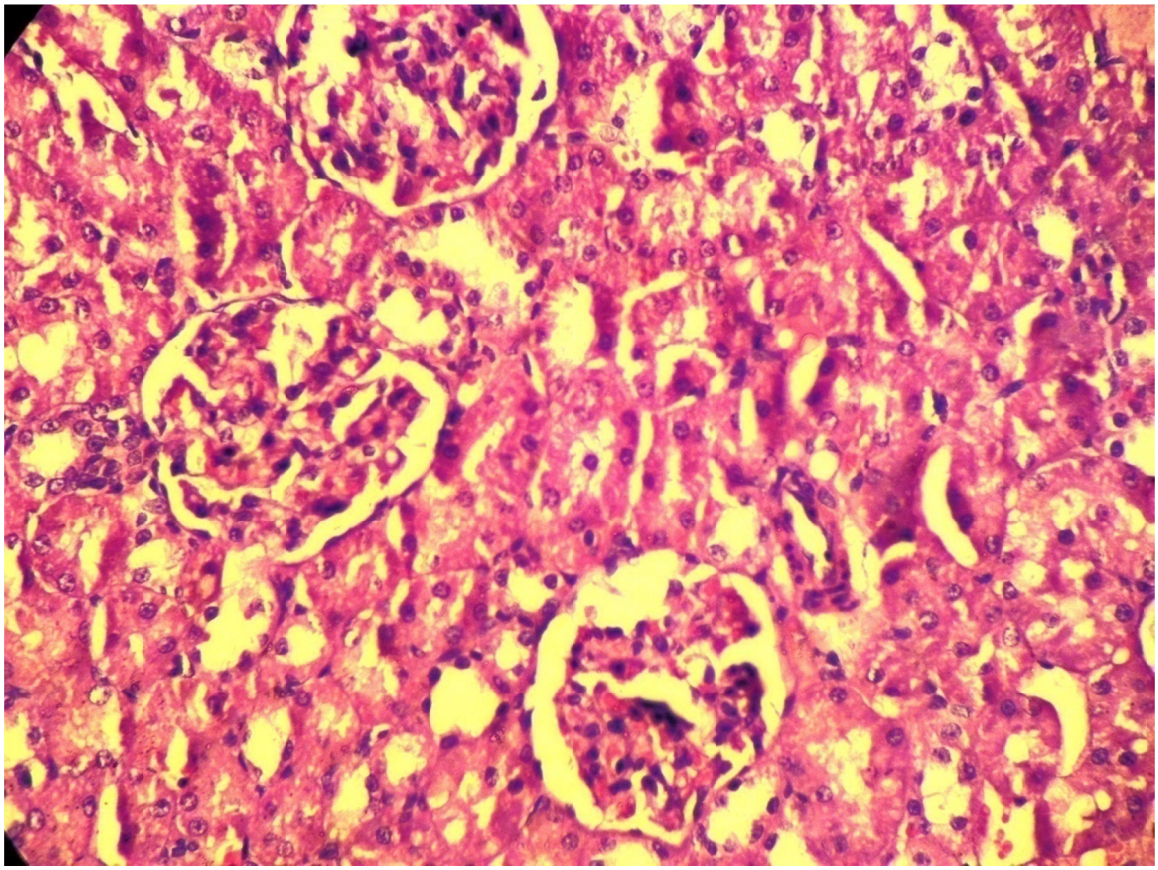


Figure. No: 17

Kidney Section of GP_4(Ascorbic acid 10mg/kg/mice)

Shows mild renal tissue damage. No evidence of heamorrhage,glomerular atrophy

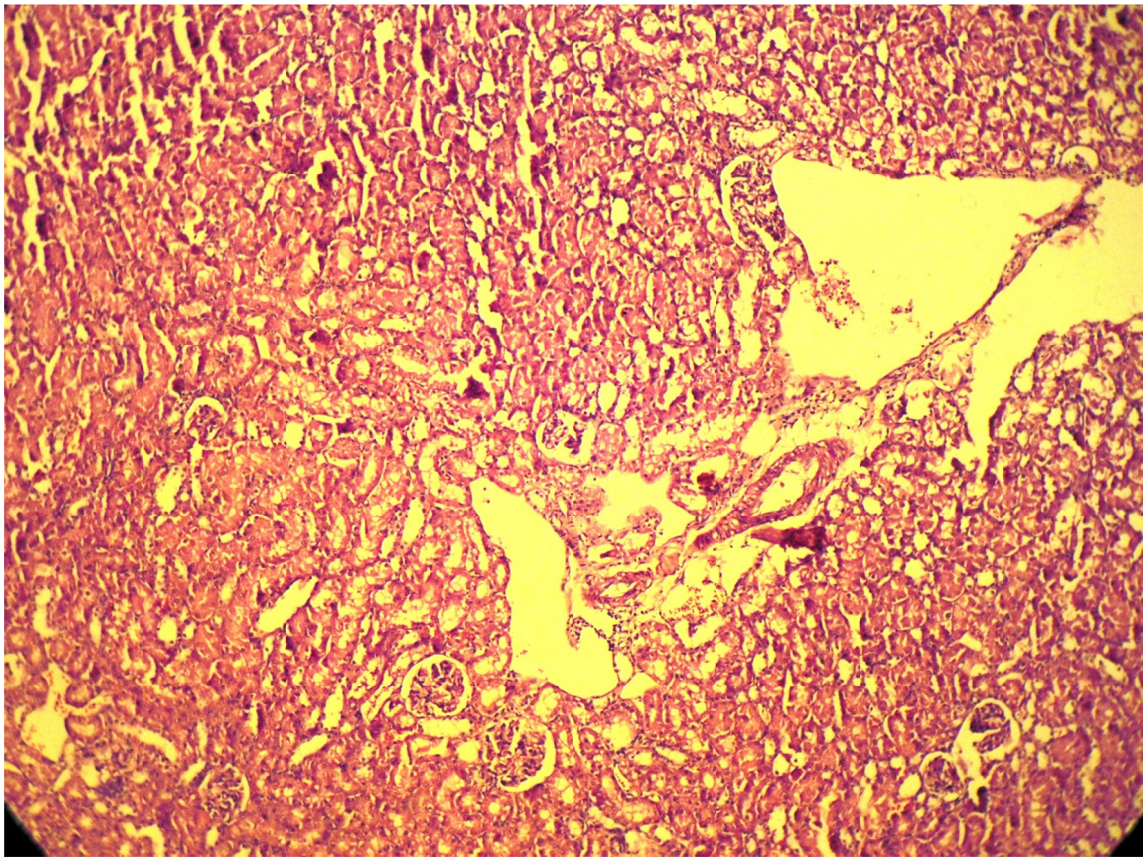


Figure. No: 18

Kidney Section of GP_5 (Ifosfamide+Ascorbic acid 10mg/kg each per mice)

Shows no renal tissue damage. No evidence of heamorrhage, glomerular atrophy

CHAPTER – VIII

Discussion

DISCUSSION

In the studies related with the assessment of antitumor activity of various drugs, ascites Dalton's lymphoma has been commonly used as an important murine experimental tumor model [73,74,75,76]. As this malignant Dalton's Lymphoma is related with the lymphocytes (mainly T-lymphocytes), it may also be helpful in correlating some human cancer related with lymphocytes such as precursor T-cell leukemia/lymphoma, non-Hodgkin lymphoma, Burkitt's lymphoma, BCL , Follicular lymphoma, MALT lymphoma etc. Ifosfamide and its derivates are alkylating agents which have been used against various malignancies particularly chronic lymphocytic leukemia (CLL)[77,78]. Ifosfamide has also been used in combination with other agents such as fluderabine [79], 2-(morpholin-4-yl)-benzo[h]chomen-4-one [80], levamisole [81] against various cancers. The host survival data from the present studies indicate significant increase in survivability of the tumor bearing mice treated with Ascorbic acid (AA) plus Ifosfamide, as compared to the group of mice treated with either agent alone, suggesting additive/ synergistic antitumor activity of AA and Ifosfamide against murine Dalton's lymphoma.

Several mechanisms have been elucidated to explain the AA-mediated enhancement in the antitumor activity and apoptosis of anticancer drugs. There is also increasing evidence that AA is selectively cytotoxic to some types of tumor cells, functioning as a pro-oxidant, rather than anti-oxidant. It has been reported that AA induces apoptosis with the generation of GSH oxidation and H_2O_2 accumulation in acute myeloid leukemia (AML) cells. Induction of apoptosis in AA-treated AML cells involved a dose-dependent increase of Bax protein, release of cytochrome C from mitochondria to cytosol, activation of caspase 9 and caspase 3, and cleavage of poly (ADP-ribose) polymerase [82]. The pro-oxidant activity of ascorbic acid is due to its ability to redox-cycle with transition metal ions, and thereby stimulates the formation of species such as superoxide, hydrogen peroxide and hydroxyl radicals.

Development of Ifosfamide induced blood related toxicity such as myelosuppression- pancytopenia, anaemia, thrombocytopaenia and/or leukopaenia[77,83,84] is another problem. Depletion in erythrocytes leads to iron

deficiency, anemia [85] and is a frequent complication of cancer diseases. Tung *et al.*[86] mentioned that the reduction in the values of blood parameters like RBC, WBC and Hb may be attributed to the hyperactivity of bone marrow, which leads to production of red blood cells with impaired integrity that are easily destroyed in the circulation. In the present study also a significant decrease in the haematological parameters i.e. RBC, WBC counts and Hb contents, were observed in tumor-bearing animals after Ifosfamide treatment (Table 5). AA plus Ifosfamide co-treatment of tumor-bearing animals caused significant recovery in these haematological values (Table 5). Combination treatment with AA plus Ifosfamide resulted in a significant (*P*_0.05) decrease and betterment in abnormal RBCs as compared to Ifosfamide alone.

The decreased life span of RBCs and anemia may be correlated with decreased blood antioxidant capacity [87]. In addition to oxygen transport, RBCs also function as conveyors of nutrients, and serve as targets for drugs, pathological factors and environmental xenobiotics [88]. Severe Ifosfamide mediated hematotoxicity and bone marrow suppression are also reported by many workers [89,90]. Ascorbate (vitamin C) is considered to be an important antioxidant in extracellular fluid including blood and protects plasma lipids from peroxyl radicals mediated peroxidative damage[91]. Ascorbic acid has been reported to cause protective effect against hematological toxicity induced by chlorpyrifos[92] and carbamazepine[93] also in rats. The vitamins (C and E), supplementation has also been found to be associated with reduced toxic effects of ethanol on liver weight and some blood parameters in rabbit[94].

CHAPTER – IX

Conclusion

CONCLUSION

In conclusion, it may be suggested that combination treatment of Ascorbic acid plus Ifosfamide could be very useful in enhancing Ifosfamide mediated therapeutic efficacy which involves induction of apoptosis in DL cells with higher apoptotic index. Ifosfamide treatment induced hematotoxicity and renal toxicity in the host but the treatment with Ascorbic acid plus Ifosfamide showed significant decrease in these toxicities indicating a protective effect, thus, indicating differential effects of the combined treatment on the cancer cells and other tissues of the host.

CHAPTER – X

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